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DEVELOPMENT OF MULTI-CELLULAR HUMAN LUNG MODELS TO STUDY INFLAMMATORY MECHANISMS AND CELL-CELL INTERACTIONS

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Cover: Primary bronchial epithelial cells at air-liquid interface and co-cultured with macrophage on apical side. From left to right: Ciliated cell, Club (Clara) cell, Mucus producing cell and Basal cell. Upside: Macrophage.

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DEVELOPMENT OF MULTI-CELLULAR HUMAN LUNG
MODELS TO STUDY INFLAMMATORY MECHANISMS
AND CELL-CELL INTERACTIONS
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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This thesis is dedicated to my family

Stay Hungry. Stay Foolish

ABSTRACT

It is well known that exposure to toxicants in the environment is associated with a wide range of health effects. The airway epithelium which forms the first line of defense, plays critical roles in the defence against inhaled particles, pathogens and toxic agents. Therefore, good and valid human airway models need to be developed to study pathophysiological mechanisms of pulmonary toxicity mediated by xenobiotics. In this thesis, unique systems which combine different human airway cell models (*ex vivo* and *in vitro*) and different exposure methods were developed. After exposure, inflammatory and oxidative stress responses, interaction between different cell types, as well as the roles of Toll-like receptors (TLR) were addressed.

In Paper I, we found that TLR ligands increased the release of pro-inflammatory cytokines and chemokines in alveolar macrophage (AMQ) from both healthy non-smokers and smokers with and without chronic obstructive pulmonary disease (COPD), and this induction was attenuated by co-stimulation with glucocorticosteroids. Glucocorticosteroids alone or combined with TLR ligands upregulated the TLR2 expression in AMQ from smokers with and without COPD. Hence glucocorticosteroids both exert an anti-inflammatory effect by inhibiting the inflammatory response and improvement of the host defense response by increasing the TLR2 expression. This may be a contributing mechanism for the positive effect of glucocorticosteroids in the treatment of acute exacerbation caused by microorganisms in COPD.

In Paper II-III, we succeeded in establishing human bronchial epithelial models (primary bronchial epithelial cell cultured at air-liquid interface (PBEC-ALI) with/without fibroblasts). In Paper IV, we built multi-cellular human bronchial models (PBEC-ALI co-cultured with macrophages (MQ); PBEC-ALI/MQ). After airlifting, the bronchial epithelial cells differentiated into ciliated cells, basal cells, mucus producing cells, and Club (Clara) cells which all are cell types present bronchial epithelium *in vivo*. Interleukin 13 (IL-13) induces mucus producing cell metaplasia and hyperplasia and was used to build up chronic bronchitis-like models including an increased number of mucus-producing cells. By combining these models with a controlled aerosol exposure system (XposeALI), we developed an *in vitro* testing strategy to mimic *in vivo* conditions, a strategy which substantially reduces the need for animal models.

In Paper II and III, we demonstrated that the induced inflammatory and oxidative stress responses and altered tissue injury/repair might be responsible for palladium/carbon nanoparticle mediated pulmonary toxicity. In addition, by comparison of inflammatory and oxidative stress response between normal and chronic bronchitis-like models, we found the latter to be more sensitive to particles exposure than the normal models. These results indicated that a pre-existing condition like chronic bronchitis, might lead to an increased risk of nanoparticle mediated health effect indicating an enhanced susceptibility to air pollution exposure in individuals with chronic lung disease compared with healthy subjects.

In Paper IV, we showed that diesel exhaust particles, in PBEC-ALI models, induced inflammatory and oxidative stress responses that were attenuated in the presence of MQ. In PBEC-ALI/MQ, diesel exhaust particle exposure increased the mRNA expression of M2-MQ markers which was not observed in mono-cultures (PBEC-ALI or MQ). These findings indicate that the cross-talk between epithelial cells and MQ, together with particle exposure can drive MQ polarization towards M2-subtype. Therefore, interactions between PBEC and MQ play an important role in resolution of the inflammatory response upon particle exposure.

Taken together, this thesis indicates the role of TLR mediated inflammatory response and oxidative signaling pathway as well as cell-cell interactions after exposure to various toxic components on different lung cell models related to COPD and chronic bronchitis.

LIST OF SCIENTIFIC PAPERS

The thesis is based on the following papers, referred to by their Roman numbers

- I. **Ji J**, von Schéele I, Billing B, Dahlén B, Lantz AS, Larsson K, Palmberg L. Effects of budesonide on toll-like receptor expression in alveolar macrophages from smokers with and without COPD.
Int J Chron Obstruct Pulmon Dis. 2016; 11: 1035–1043.
- II. **Ji J**, Hedelin A, Malmlöf M, Kessler V, Seisenbaeva G, Gerde P, Palmberg L. Development of Combining of Human Bronchial Mucosa Models with XposeALI® for Exposure of Air Pollution Nanoparticles.
PLoS One. 2017 Jan 20; 12(1).
- III. **Ji J**, Ganguly K, Mihai X, Jitong S, Malmlöf M, Gerde P, Upadhyay S*, Palmberg L*. Exposure of normal and chronic bronchitis-like mucosa models to aerosolized carbon nanoparticle: Comparison of pro-inflammatory and oxidative stress response. *Equally contributed (Manuscript).
- IV. **Ji J***, Upadhyay S*, Xiong X, Malmlöf M, Sandström T, Gerde P, Palmberg L. Multi-cellular Human Bronchial Models Exposed to Diesel Exhaust Particles: Assessment of Inflammation, Oxidative Stress and Macrophage Polarization. *Equally contributed.
Particle and Fibre Toxicology. 2018 May; 15:19.

Additional publications not included in the thesis

- V. **Ji J**, von Schéele I, Bergström J, Billing B, Dahlén B, Lantz AS, Larsson K, Palmberg L. Compartment differences of inflammatory activity in chronic obstructive pulmonary disease.
Respir Res 2014; 15:104.
- VI. Feliu N*, Kohonen P*, **Ji J**, Zhang Y, Karlsson HL, Palmberg L, Nyström A, Fadeel B. Next generation sequencing reveals low-dose effects of cationic dendrimers in primary human bronchial epithelial cells. *Equally contributed.
ACS Nano. 2015 Jan 27; 9(1):146-63.
- VII. Sundblad BM*, **Ji J***, Levänen B, Lidén C, Larsson K, Palmberg L, Lindén A. Extracellular cadmium in the bronchoalveolar space of long-term tobacco smokers with and without COPD and its association with inflammation. *Equally contributed.
Int J Chron Obstruct Pulmon Dis. 2016; 11: 1005–1013.
- VIII. Lukic A, **Ji J**, Idborg H, Samuelsson B, Palmberg L, Gabrielsson S, Radmark O. Pulmonary epithelial cells, and their exosomes, metabolize myeloid cell derived leukotriene C4 to leukotriene D4.
J Lipid Res. 2016; Sep;57(9):1659-69.
- IX. Bao AH, Che Fru K, Bozinovski S, **Ji J**, Gregory JA, Georén SK, Adner M, Cardell L, Lindén A. Recombinant Human IL-26 Facilitates the Innate Immune Response to Endotoxin in the Bronchoalveolar Space of Mice in Vivo.
PLoS One. 2017 Dec 5;12(12).
- X. Gregory JA, Kemi C, **Ji J**, Karén J, Delin I, Gerde P, Pieper MP, Adner M, Larsson K. Effects of tiotropium bromide on airway hyperresponsiveness and inflammation in mice exposed to organic dust.
Pulm Pharmacol Ther. 2018 Feb; 48:203-210.

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LIST OF ABBREVIATIONS

3D	3 Dimension
3R	Replacement, reduction and refinement
ALI	Air-liquid interface
AMQ	Alveolar macrophages
AP1	Activator protein 1
ATI/ATII	Alveolar type I/ II cells
BAL	Bronchoalveolar lavage
BM/AM	Basal/ apical lavage medium
Bud	Budesonide
CC10	Club (Clara) cell protein 10
CD	Cluster of differentiation
CNP	Carbon nanoparticles
COPD	Chronic obstructive ppulmonary disease
CXCL	C-X-C motif chemokine
DEP	Diesel exhaust particles
ECM	Extracellular matrix
FEV ₁	Forced expiratory volume in one second
FOXJ1	Forkhead box protein J1
FVC	Forced vital capacity
GPx	Glutathione peroxidase
GR	Glucocorticoid receptor
GST	Glutathione S-transferases
H&E	Hematoxylin and eosin
HMOX	Heme oxygenase
ICS	Inhaled corticosteroids
IL	Interleukin
KEAP-1	Kelch-like ECH-associated protein 1
KRT5	Keratin5
LABA	Long-acting β adrenoceptor agonists
LPS	Lipopolysaccharide
M1/M2	Type1/2 macrophages
MAPK	Mitogen-activated protein kinases

MMP	Matrix metalloproteinases
MQ	Macrophage
MRC1/MRC2	Mannose receptor C-type 1/2
MRC-5	Human fibroblasts cell line
MUC5AC	Mucin 5AC
MyD88	Myeloid differentiation primary-response protein 88
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	NF-E2 related factor 2
PAMP	Pathogen-associated molecular patterns
PAS	Periodic acid–Schiff
PBEC	Primary bronchial epithelial cells
PBEC-ALI	PBEC culture at air-liquid interface
PBEC-ALI/MQ	PBEC-ALI co-culture with macrophage on apical side
PBEC-ALI/CB	PBEC-ALI treated with 1ng/ml IL-13
Pd-NP	Palladium nanoparticles
PGN	Peptidoglycans
PM	Particulate matter
PRR	Pattern recognition receptor
RETNLA	Resistin-like molecule alpha
ROS	Reactive oxygen species
SEM	Scanning electron microscope
SOD	Superoxide dismutase
TEER	Transepithelial electrical resistance
TEM	Transmission electron microscope
THP-1	Human monocytic cell line
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TNF	Tumor necrosis factor

1 INTRODUCTION

With the rapid development of industrialization, death from environmental degradation has become a huge threat to the public health. Pollution is one of the leading environmental hazards that affects our daily lives. According to a report from World Health Organization (WHO), in 2012, around 7 million people died and one in eight of the total global deaths is directly linked to air pollution exposure. This report in 2014 confirmed that air pollution represents the world's largest signal environmental health risk[1]. Air pollution involves inhalation exposure to particulate matter (PM), gases and fumes including metal fume and may contribute to the development of cardiopulmonary diseases and lung cancer[1]. However, the mechanism of xenobiotics induced potential lung toxicity remains relatively unknown primarily due to the lack of efficient test systems which can mimic human exposure scenarios to aerosolized air pollutants.

Therefore, in this thesis, we developed different cell models including single and multiple cell types and combined with submerge/aerosol exposure. These models were used to identify the cross-talk between cells as well as the Toll-like receptor mediated inflammatory and oxidative signaling pathway after exposure to various particles existing in air pollution.

1.1 THE RESPIRATORY SYSTEM

The human respiratory system is a biological system responsible for exchanging carbon dioxide and oxygen. For an adult at rest, the normal respiration rate is 12-20 breaths/min and normal minute volume is approximately 7.5 L/min. Inhaling air enters into the respiratory system through the nose or mouth and passes through the trachea, bronchi and bronchioles and finally reaches the alveoli. The respiratory system can be divided into upper and lower airways. The upper airway consists of the nasal cavities, pharynx and larynx. The lower airway which divides into two main bronchi after which there is dichotomous divisions of the airways down to terminal and respiratory bronchioles, alveolar ducts and alveoli. The airways are lined with ciliated epithelial cells which functioned as a mechanical barrier. The synchronized cilia beating transports mucus produced by mucus producing cells located in the airway epithelium or submucosal glands towards pharynx. This mucociliary clearance clears the airways from inhaled particles which are trapped in the mucus and prevent accumulation of mucus in the lung.

The alveolar epithelial cells consist of flat alveolar type I (ATI) cells (90% of the alveolar surface) and cuboidal alveolar type II (ATII) cells (10% of the alveolar surface). Both of them are not ciliated and have direct contact with the capillary endothelium where the gas exchange occurs. Figure1 indicated the anatomy of respiratory system (Figure1A) and cellular transition of airway and alveolar epithelial layer (Figure1B).

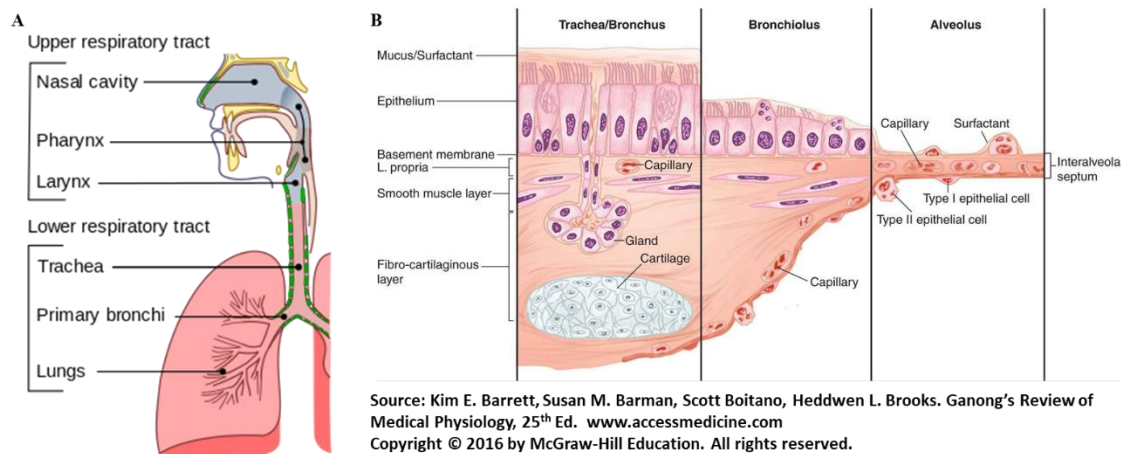


Figure 1. Anatomy of respiratory system and cellular transition of airway and alveolar epithelial layer

Cigarette smoke or exposure to pollutants impair the mucociliary clearance and increase mucus secretion which induce the risk of airway infections[2, 3]. The abnormalities in mucus production and clearance contribute to airway obstruction, lung function decline and lead to respiratory disease. Chronic respiratory diseases are diseases affecting airways which hundreds of millions of people suffered from. The most common chronic respiratory diseases are chronic obstructive pulmonary disease (COPD), chronic bronchitis and asthma.

1.1.1 Chronic Obstructive Pulmonary Disease (COPD)

Chronic Obstructive Pulmonary Disease (COPD) is a multi-component disease with inflammation in the large/small airways (bronchitis/ bronchiolitis) and lung parenchyma destruction which lead to the abnormal airway space enlargement (emphysema)[4]. It affects up to 10% of the population world-wide and it is estimated to be the third leading cause of death in 2020[5, 6]. During the last decade moderate and severe COPD is also recognized as a systemic disease with signs of systemic inflammation[7] and a massive co-variation with other conditions such as cardiovascular disease, asthma, osteoporosis, skeletal muscle dysfunction, cognitive dysfunction, and diabetes[8, 9].

In this thesis, the classification of COPD refers to Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria[10] (table 1). The classification of severity is based on lung function as assessed by spirometry.

Table 1. Spirometric Classifications in Different Stages of COPD[10]

Post-bronchodilator lung function measurements and FEV ₁ /FVC < 70% is prerequisite for all the stages	
Stage I: Mild COPD	FEV ₁ >80% predicted
Stage II: Moderate COPD	50% < FEV ₁ < 80% predicted
Stage III: Severe COPD	30% < FEV ₁ < 50% predicted
Stage IV: Very Severe COPD	FEV ₁ < 30% predicted or <50% predicted with chronic respiratory failure or clinical signs of heart failure, low BMI or chronic mucus hyper secretion

FEV₁: forced expiratory volume in one second. FVC: forced vital capacity, BMI: body mass index.

Tobacco smoking is the most important risk factor of COPD. Although not all cigarette smokers will develop the disease, approximately 50% of the smokers who reach the age of 75 years develop COPD[11]. Also exposure to gases, dust or fumes in the workplace increase the risk of COPD. In general population, approximately 15% of all cases of COPD are due to occupational exposure including coal or metal mining, construction and farming biomass[12]. Long-term exposure to air pollution contributes to the development of COPD[13], and short-term exposure to air pollutions may cause acute exacerbations in subjects who suffer from the disease[14]. Alpha-1 antitrypsin (AAT) deficiency is strongly associated with COPD, especially in smokers. Up to 5% of people with COPD have been estimated to have AAT deficiency[15].

The COPD is associated with irreversible damage of the lungs. Acute exacerbations of COPD drive impaired health related quality of life, increase symptoms, increase the risk of infections, increase lung function decline over time, increase the risk of hospitalization and increase mortality in patients. The goals of COPD treatment include relief of symptoms, limitation of disease progression, prevention of exacerbations and reduction of mortality[16]. Smoking cessation is the most effective treatment for COPD, and it is the only proven way of influencing rate of decline in lung function with time[17]. Physiotherapy is an important component in the treatment of COPD as is pharmacological treatments including long acting bronchodilators (β_2 -agonists (LABA) / anticholinergic agents) and glucocorticosteroids.

Inhaled corticosteroids (ICS), in particular when combined with long-acting beta-2-agonists have been shown to improve health status and reduced the exacerbation rates[18]. Glucocorticosteroids like budesonide (Bud) is a class of corticosteroids and widely used for the treatment of COPD. As an anti-inflammatory drug, it can bind to intracellular glucocorticoid

receptors (GRs) and co-operate with transcription factors to downregulate pro-inflammatory genes and/or upregulate anti-inflammatory genes transcriptions[19]. The combination of glucocorticosteroids and LABA have been proved to be of greater beneficial effect than each of them alone[18]. This synergistic effect is due to that on one hand glucocorticosteroids can modulate the function of β_2 -adrenoceptors thereby preventing the tolerance induced by β_2 -agonists[20], on the other hand LABA seems to facilitate the GRs translocation into the nucleus[21].

Anticholinergic agents are the drugs of choice to treat COPD. Recent studies[22] have demonstrated that beneficial clinical effects of anticholinergics in COPD are mediated, not only by blocking parasympathetic nerve impulses in the bronchial smooth muscle, but also by interacting with inflammatory cells.

1.1.2 Chronic bronchitis

Chronic bronchitis is a disease associated with structural changes of large and medium-size airways which are mainly caused by tobacco smoking. Some other risk factors such as air pollution and occupational airborne exposures have been also identified[23]. The definition of chronic bronchitis is: daily chronic coughing and sputum production for at least 3 months a year for 2 years in a row. It is characterized by metaplasia, hypoplasia and a much more peripheral distribution of goblet cells compare with healthy non-smokers, as well as increasing submucosal gland size which leads to increased mucus production[24]. This in turn decrease mucociliary clearance and destroyed cilia, broken epithelial barrier and thickening of the bronchial wall[25]. Chronic bronchitis is a commonly neglected co-morbidity of COPD, one that further increases morbidity and mortality in an independent manner[26].

1.2 CELLS INVOLVED IN HOST DEFENCE

In human body, the innate and adaptive immune system work as the defense against a variety of pathogens and infections. The respiratory tract host defense is provided by immune effector cells (alveolar macrophages, neutrophils and dendritic cells) and structural cells (epithelial cells and fibroblasts). They cooperatively participate in the orchestration of immune responses as they all express pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), nucleotide binding and oligomerization domain-like receptors (NOD or NLRs), retinoic acid-inducible gene 1-like receptors (RLRs) on their cell surface. The PRRs recognize pathogen associated molecular patterns (PAMPs) on bacterial and viral pathogens[27, 28] or danger-associated molecular patterns (DAMPs) from damage tissue.

1.2.1 Macrophage

Macrophages (MQ) is a population of phagocytic cells which are derived from blood monocytes, and play important roles in non-specific defense mechanisms involved in the defense against external insults[29]. They are able to modulate the adaptive immune responses of the host by secreting and responding to a variety of effector molecules[30]. Two major polarization states of macrophages have been described: the classically active type1 (M1) and alternatively activated type 2 (M2). M1 macrophages are characterized by high production of pro-inflammatory cytokines, reactive nitrogen and oxygen intermediates (RNIs and ROIs) as well as by a strong ability to mediate resistance to pathogens and microbicide properties[31]. Lipopolysaccharides (LPS), IFN- γ and TNF- α stimulate the classical pathway of M1 activation[32]. In contrast, M2 macrophage are characterized by secreting anti-inflammatory cytokines with limited production of pro-inflammatory cytokines[33] and they are considered to have wound-healing properties with induction of scavenger, mannose receptor 1 or 2 (MRC-1/MCR-2) and adipokine resistin-like molecule alpha (Retnla) expressions[34]. Interleukin (IL)-4 and IL-13 or immune-regulatory signals like IL-10 can drive the polarization of macrophage skew towards the M2 phenotype[32]. Phenotypic alteration of MQ is considered to play a pivotal role in the pathogenesis of chronic airway diseases and has potential implications for the treatment of chronic respiratory diseases like COPD and asthma[35].

1.2.2 Bronchial epithelial cells

Airway epithelium plays a protective role against inhaled xenobiotics. It is pseudostratified in the large airways and becomes columnar and cuboidal in the small airways[36]. *In vivo*, human bronchial epithelium consists of ciliated cells (50-70 %), basal cells (30 %), mucus producing cells (25 %) and Club (Clara) cells (11 %)[37].

Ciliated cells are usually equipped with up to 300 cilia per cell and contain numbers of energy-producing mitochondria close to their apical surface[38]. Matured cilia shows the ultra-structure of axoneme with two central singlet and nine outer doublet microtubules which can be stained with anti-acetylated alpha tubulin antibody[39]. With the cilia beating, the mucociliary clearance is performed by moving the mucus continuously up to pharynx, thus inhaled agents which are embedded in the mucus are gradually cleared away.

Mucous (goblet) producing cell is membrane-bound and include acidic mucin granules, which can secrete mucus that increase the physical barriers to trap inhaled agents[40]. Mucin 5AC (MUC5AC) is one of the major gel-forming secreted mucins expressed by mucous producing cells[41]. Under normal conditions mucus protects the airway epithelium and is an important

part of the mucociliary clearance defense. However, abnormalities in mucus secretion or clearance can engender respiratory disease. For instance, chronic bronchitis which is usually characterized by chronic hypersecretion of airway mucus and further lead to airway obstruction, lung function declination and mortality[42, 43]. Both IL-4 and IL-13 can induce metaplasia or hyperplasia of mucous producing cells and can also increase MUC5AC secretion/expression[44], which are the characteristic features of chronic bronchitis.

Club (Clara) cells usually have stem cell properties and regard as progenitor cells for ciliated cells[45]. Club cells can produce club cell protein also known as: uteroglobin, club cell protein 16 (CC16) or club cell protein 10 (CC10)[45]. *Club cell protein* has an anti-inflammatory effect and its expression in bronchial epithelium of COPD patients and smokers with chronic bronchitis has been shown to be significantly decreased compared with healthy controls or smokers without chronic bronchitis, respectively[46, 47].

Basal cells are located beneath other epithelial cells and firmly attached to the basement membrane. They are also considered as stem cells and can give rise to stroma or columnar epithelial cell[48].

Bronchial epithelial cells at air-liquid interface (ALI)

Traditionally, *in vitro* studies have been performed by culturing the cells submerged where medium and target agents were added on top of the cells to cover the cells. For primary contact organs such as the lung, exposure to external agents occurs at ALI implicating that the submerged condition does not resemble the *in vivo* situation associated with exposure in human respiratory. Moreover, the bronchial epithelial cell cultures under submerged conditions develop a squamous phenotype, while under ALI conditions the epithelial cells differentiate into pseudostratified epithelial layer which contains ciliated cells mucus producing cells, club cells and basal cells. Thus, ALI culture mimic is similar to the *in vivo* situation. Now more and more ALI cultures are commercially available at the market (MucilAirTM-HF and EpiAirwayTM) and different ALI exposure systems have been developed to gain an insight of inhalation toxicology research[49-51].

1.2.3 Crosstalk between macrophage and bronchial epithelial cells

The cooperation between macrophages and epithelial cells are important as they both work within the first line of defense against inhaled toxic agents on both airways and alveolar levels. Previous studies have shown that bronchial epithelial cells[52] recognize conserved PAMPs through apical TLRs including TLR2 and TLR4, and subsequently induce the secretion of pro-inflammatory cytokines and chemokines, which in turn stimulate recruitment and activations

of phagocytes like macrophage to the site of infections[53]. Macrophages in itself is also equipped with TLRs that recognize a wide spectrum of inhaled particles or pathogens and consequently contribute to the cytokines production. The synthetization of cytokines from both cell types may induce inflammation and contribute to the development of airway disease like COPD and asthma[54]. Interestingly, it has also been reported that bronchial epithelial cells can regulate the TLRs sensitivity to avoid the risk of continuous inflammation and limit the release of pro-inflammatory cytokines by innate immune cells like macrophages[55].

1.3 TOLL-LIKE RECEPTORS

Toll-like receptors (TLR) are a class of PRRs enabling cells to recognize PAMPs from microorganisms or DAMPs from tissue injury. Structurally, TLRs are type I transmembrane glycoprotein on the basis of: an amino (N)- terminal extracellular domain consist by horseshoe-like leucine rich repeats (LRRs); a single transmembrane region; a carboxyl (C)-terminal intracellular Toll/interleukin-1 receptor (TIR) domain contains three highly homologous regions (box 1, 2 and 3)[56]. To date, 10 human and 12 mouse TLRs have been identified. In human beings, TLRs can be subdivided into two groups depending on the site of expression. One type is expressed at the cell surface and detect PAMPs in microbial membranes and flagellin (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10). The other type is expressed within the intracellular compartments and are activated by PAMPs in nucleic acids derived from bacterial and viral pathogens (TLR3, TLR7, TLR9 and TLR10)[57].

1.3.1 TLRs signaling

TLRs signaling can be classified as either myeloid differentiation primary-response protein 88 (MyD88)- or TIR-domain-containing adaptor-inducing interferon- β (TRIF)- dependent pathways[58].

MyD88 dependent pathway

Upon activation, MyD88 can directly bind to TIR domains of TLR or with the help of MyD88 adaptor-like (MAL)/ TIR domain-containing adaptor protein (TIRAP). The association of MyD88 and interleukin-1 receptor-associated kinase (IRAK) 4 induces phosphorylation of IRAK1. Activation of IRAKs recruits TNF receptor associated factor (TRAF) 6 to the receptor. Then IRAK1/TRAF6 dissociates from receptor and form a complex with transforming growth factor beta-activated kinase1 (TAK1), TGF-beta activated kinase (TAB) 1 and TAB2. Then TAK1 become phosphorylated and this process activates I κ B kinase (IKK) complex or mitogen-activated protein kinas (MAPK). The activation of both kinases allowing nuclear

factor kappa-light-chain-enhancer of activated B cells (NF- κ B) or activator protein 1 (AP-1) to translocate into nucleus and induce the expression of target genes.

TRIF-dependent pathway

Upon activation, TRIF directly or with the help of TRIF-related adaptor molecular (TRAM) associated to TRAF6 or TRAF3. The engagement of TRAF3 can trigger TANK binding kinase 1 (TBK1) mediated interferon regulatory factor 3 (IRF3) activation and increased type I interferon (IFN)s production.

Negative regulation of TLR signaling

Excessive activation of TLR signaling may disrupt the immune homeostasis and initiate inflammatory and autoimmune diseases. Therefore, a negative control of TLR signaling seems very important to keep the balance of the immune system. There are several mechanisms involved in this negative regulation[59]. The major mechanism is to reduce the function and expression of TLR. Another mechanism is including intracellular negative regulators like IRAK-M, MyD88 short, SOCS1 *etc.*[60].

1.3.2 TLR2 and TLR4

Because of their ability to recognize a variety of pathogenic ligands (both endogenous and exogenous), TLR in general but especially TLR2 and TLR4 have received great attention since they were discovered.

TLR2 is localized on the cell surface, and form heterodimers with TLR1 or TLR6 to identify a wide range of ligands including peptidoglycan (PGN) and lipoteichoic acid (LTA) from Gram-positive bacteria, bacterial lipoproteins and lipopeptides, envelope proteins from virus *etc.*[61]. *TLR4* is the first TLR to be characterized and well-studied[62]. Several ligands for instance lipopolysaccharide (LPS) from Gram-negative bacteria and fusion and envelope proteins from virus stimulate TLR4[63]. LPS is one of the immuno-stimulatory membrane components that elicits a strong systemic inflammation. LPS is directly captured by a soluble shuttle protein, LPS binding protein (LBP), and by that facilitates LPS binding to cluster of differentiation 14(CD14) on the cell surface[64]. CD14 is a glycosylphosphatidylinositol-anchored protein on the surface of monocyte/macrophage which delivers the LPS-LBP complex to TLR4/MD-2 receptor and realize LPS recognition[65]. As MD-2 is in a soluble form and not covalently bound to TLR4, it can also independently be associated with LPS without TLR4 present[66]. In addition to binding of exogenous ligands, TLR2 and TLR4 also interact with endogenous ligands formed under stress or tissue injury. Heat shock proteins

(HSP) like HSP60, HSP70, HSP90, GP96 and HSP22 are endogenous ligands for TLR2 and TLR4[67, 68]. The high-mobility group box 1 (HMGB1) and small molecular weight fragments of hyaluronic acid (HA) from damaged cells are recognized by TLR2 and TLR4 and triggers inflammation[69, 70]. Upon stimulation, TLR2 signaling go through the MyD88-dependent pathway and TLR4 activate both MyD88- dependent and TRIF- dependent pathways (Figure 2 left).

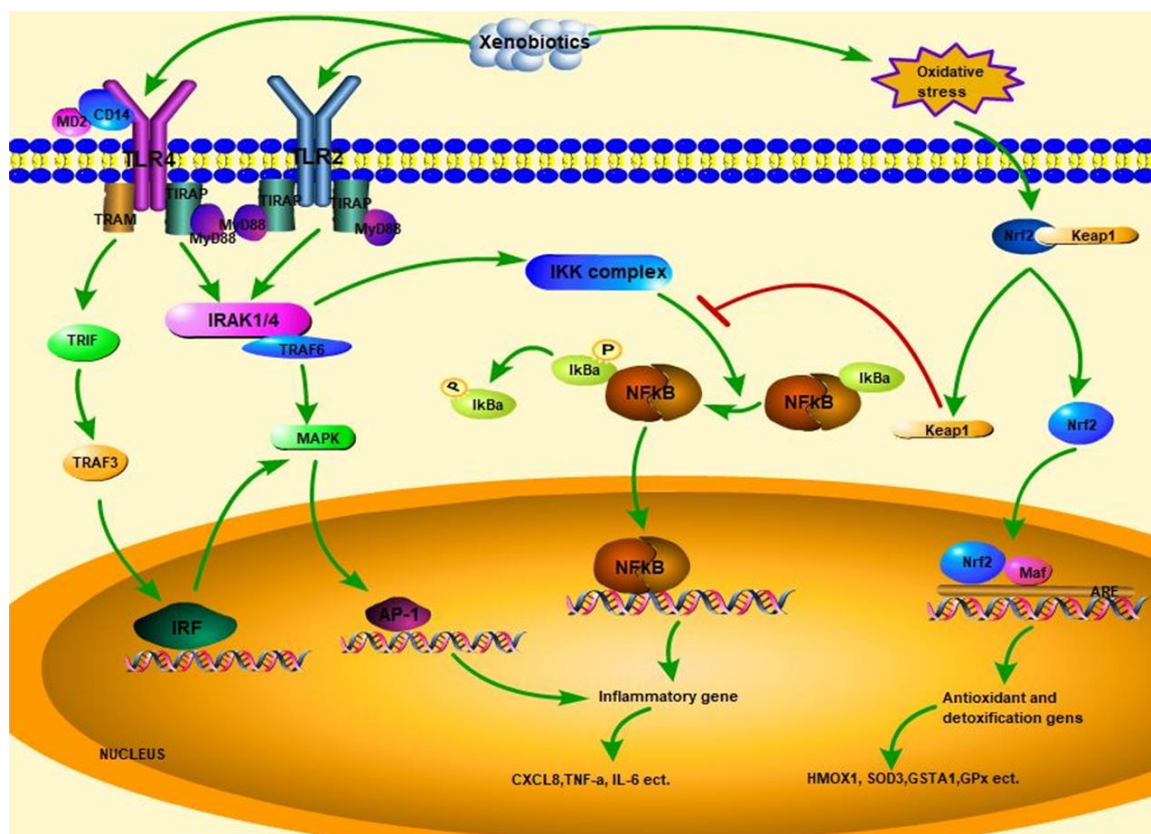


Figure 2. TLR2 /TLR4 mediated inflammatory (left) and oxidative stress (right) signaling pathways after exposure to various xenobiotics.

In previous studies it has been found that TLR2 mRNA expression was lower in sputum neutrophils and alveolar macrophages from smokers with COPD than in healthy controls[71, 72]. Further it has been shown that TLR4 mRNA and protein expression were down-regulated upon cigarette smoke extracts exposure in an airway epithelial cell line[73]. All of these findings confirmed the important roles of TLR2 and TLR4 in host defense mechanisms.

1.4 PARTICULATE MATTER

Particulate matter (PM) is one of the major health risk components in air pollutions. It is a mixture of solid and liquid particles suspended in air which with a great variation of particle size, number, composition, and origin. According to aerodynamic diameter, PM is generally

classified into 3 subtypes: PM₁₀ (coarse particles, median aerodynamic diameter (MAD) $\leq 10\mu\text{m}$), PM_{2.5} (fine particles, MAD $\leq 2.5\mu\text{m}$) and UFP (ultrafine particles, MAD $\leq 0.1\mu\text{m}$). Long-term exposure to PM increases morbidity and mortality of many diseases such as cardiovascular diseases, cancer and pulmonary diseases[74, 75]. For instance, people who are living near a major road with chronic exposure to PM₁₀ showed a high risk of development of COPD and lung function loss[76].

1.4.1 Nanoparticles

The PM with at least one geometric dimension smaller than 0.1 μm are known as nanoparticles[77]. Although humans have been exposed to nanoparticles for a long time, nowadays inhalational exposure to nanoparticles is of great concern not only from the occupational point of view but also for the environmental daily exposure to airborne nanoparticles.

Palladium nanoparticles (Pd-NP)

The Pd-NP have a size around 10 nm. The Pd-NP possess high chemical activity and are broadly used as catalysts in automotive and in chemical or processing industries. Additional use in electrical equipment, jewelry and dental appliances also contribute to environmental Pd-NP load[78]. Due to the small size, they result in a relative stable aerosol that could reach down to the alveoli in the lungs, but deposition may also occur higher up in the airway tree particularly at the bifurcations[79].

Carbon nanoparticles (CNP)

The CNP which have the size less than 100 nm. They are generated by incomplete fuel and diesel combustion, as well as in connection to indoor cooking or open air fire[80-82]. They are also involved in manufacturing of black ink, in paint, plastic, and reinforcing agents for rubber goods[83]. The concern of the risk of CNP arises because of their large effective surface area, great deposition rate and high toxicity[84, 85]. Similar to Pd-NP, CNP is deposited throughout the whole respiratory system and may cause unwanted effects both locally and systemically by crossing the alveolar-capillary barrier and enter into blood stream[86].

1.4.2 Diesel exhaust particles

Diesel exhaust particles (DEP) are a complex mixture of particles ($< 1.0\mu\text{m}$ in diameter) and form a source of PM in ambient air and occupational settings. All DEP fresh from the engine are below 1 μm , with very few particles above 500 nm in size. The outer shell of DEP consists of metals (copper/zinc) as well as polycyclic aromatic hydrocarbons (PAHs) (anthracene or

benzo[a]pyrene (B[a]P))[87]. The DEP are products of incomplete combustion of diesel fuel in engines. Although the new diesel engines have been improved to produce lower levels of PM, increased ultrafine particles and nanoparticles are still produced[88]. Due to the high efficiency robustness and low running costs, diesel engines are widely used in the mass transportation including buses, heavy duty vehicles and off-road equipment, which elevated the concern about DEP's health effects[89]. The DEP have the potential to penetrate deep down in the respiratory tract[90, 91]. Exposure to DEP in human results in marked pulmonary and systemic inflammatory responses involving different cell types[92]. Also chronic exposures is associated with long-term adverse effects, like cough, respiratory infections and lung function decline[93].

1.5 NOVEL CELL EXPOSURE SYSTEM – PRECISEINHALE: XPOSEALI

During recent decades, increasing efforts have been put on the development of systems being able to deposit aerosols on cell surfaces of cells cultured at ALI[94]. It was a breakthrough in *in vitro* research field, when techniques for exposure to aerosols including particles was possible by deposition on a cellular layer without suspending particles, in a solution as during submerged culturing. Although, some commercial cell exposure systems like ALICE, Vitrocell and Cultex® RFS[95-97] are available, the development of new technology is still under fast progress.

PreciseInhale™ exposure platform (Figure 3) which was established by Inhalation science Sweden AB is an advanced exposure system with different exposure modules. It can mimic the exposure situation *in vivo* which makes it possible to identify how inhaled particle act and affect the lung. In general, aerosol exposure of cell models requires a two-stage set-up: aerosol generator and cell exposure module. In PreciseInhale exposure platform, PreciseInhale aerosol generator can drive aerosolisation powerfully by high pressure and generates well dispersed particles through de-agglomeration[98]. The precise dosing system by the use of the Casella impactor enables an automatically dose control of the generator at each experiment. Then XposeALI® which is a newly developed cell exposure module is coupled to PreciseInhale aerosol generator. XposeALI consists of three test cups which are connected with three mass flow regulators controlled by a computer. A reverse flow pattern is used to regulate excess aerosol from the periphery to one central outlet, and subsequently collected in a filter at the end of the central outlet.

In addition to precise dosing, there are three more advantages of PreciseInhale exposure platform. Firstly, the amount of required particles is minimal implicating that only small

portions of the produced particles are needed to produce a respirable aerosol. Usually, not more than 50 mg particles can be enough for a whole study. Secondly, the deposition of particles is evenly distributed over the cell surfaces. The compressed air can disperse particles to avoid its agglomeration. Even nanoparticles which contain high cohesive energies can be well dispersed by the compressed air generated by the platform. Because of the electrostatic influence, some particles tend to stick to the insert wall. But in PreciseInhale exposure platform, the exposure hoods restrict to the exposure area available for particles of the inserts. Then the particles can be trapped inside of the exposure hoods, and directly exposed to the cell surface which avoid unwanted contamination. Thirdly, it is possible to keep the exposure time short. The high pressure of the compressed air can achieve sufficient aerosol deposition within short time. This can minimize the time for models being out of the incubator and which reduces the risk of infections and/or decreased viability. In this thesis, for all the studies, the maximal exposure time was around 7 minutes but usually a shorter exposure time was used depending of the dose.

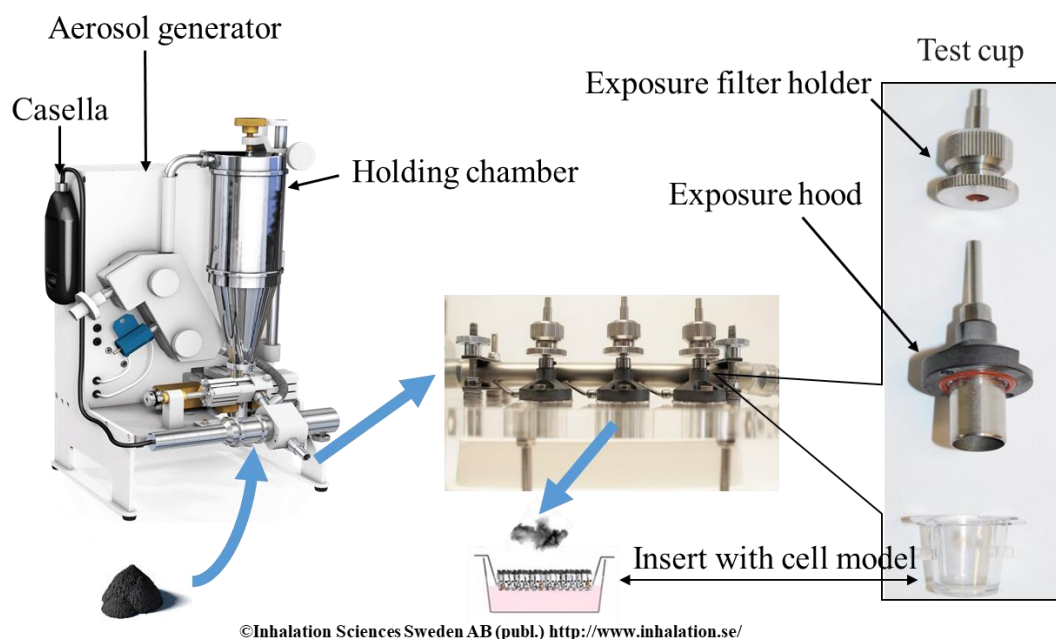


Figure 3. The PreciseInhale exposure platform with XposeALI exposure module.

1.6 INFLAMMATORY MEDIATORS

Inflammation is a complex of biological responses of tissues/organs and occurs upon exposure to harmful stimuli (damaged cells, microbial pathogens, irritants and toxic components), and controlled by different kind of mediators[99]. The classical manifestations of inflammation are pain, heat, redness, swelling and finally loss of function[100]. Cytokines with a pleiotropic activity are the regulators of host responses to inflammation. They are proteins which can be

produced by nearly all type of cells[101]. The grouping of cytokines is based on their original biological description (interleukins, tumor necrosis factors, interferons and chemokines) structural homology of their receptors (class I and II cytokines) or their roles in inflammation (pro- and anti- inflammatory cytokines)[102]. Pro-inflammatory cytokines tend to promote inflammation and make diseases worse while anti-inflammatory cytokines act to suppress the inflammation[99]. Different cytokines are signalling in different ways, they can act on the same cell that produced the cytokines (autocrine mode), affect a neighboring cell near (paracrine mode) or far from the cytokine secreting cells (endocrine mode)[102].

One of the characteristics features of COPD is inflammation in the respiratory tract and lung tissue and acute exacerbations of COPD is associated with enhanced inflammation. A variety of cell-types like structural lung cells (epithelial cells, fibroblasts) and immune cells (macrophages, neutrophils, lymphocytes) are contributing to the inflammation in COPD[103]. Also different inflammatory biomarkers varies both locally and systemically in COPD patients[7]. More than 50 cytokines have been shown to be associated with COPD, although not all of their roles have been clearly identified[104]. Some of them are of particular importance for this thesis are listed below.

Interleukin 6 (IL-6) is a cytokine with pleiotropic effects (exert acute phase/immune response and change hematopoiesis) which is secreted by T-cells, B-cells, macrophages and epithelial cells[105]. IL-6 exhibits both pro-and anti-inflammatory capacity depending on presence of other cytokines or the target cell types[106]. It can stimulate the acute phase proteins productions after exposure to different stimuli and favor the transition between acute and chronic inflammatory responses[107]. In COPD patients, especially during exacerbations, the levels of IL-6 have been found to be increased in induced sputum, BAL-fluid and serum[24].

Tumor necrosis factor alpha (TNF- α) is a key cytokine involved in inflammatory response. It is produced predominantly by activated macrophages, although it can also be secreted by other cell types including immune cells (T and B lymphocytes, neutrophils, mast cells, natural killer (NK) cells *etc.*), structural cells (epithelial cells, smooth muscle cells, fibroblasts *etc.*) and neurons[108]. TNF- α can regulate production of other cytokines, activate the extracellular matrix (ECM) expression and stimulate growth[101]. There are two isoforms of TNF α , membrane tethered a 26-kDa cytokine (mTNF- α) or a 17-kDa cytosolic soluble form (sTNF- α)[109]. TNF-receptor 1 (TNFR1, p55 receptor) and 2 (TNFR2, p75 receptor) interact with TNF- α [110]. TNFR1 is widely expressed in most cell types, and respond to both mTNF- α and sTNF- α , while TNFR2 is typically expressed with restricted levels in cells of the immune system, and only respond to mTNF- α [111]. Cell surface bound TNFR can be released to

cytoplasm by proteolytic cleavage as a response to inflammatory mediators[112]. The TNF- α regulated pathways are complex and still not fully understood. By binding to TNFR, TNF- α can activate MAPK and NF- κ B pathways, and induce death signaling[104]. This route has been implicated in the pathophysiology of many inflammatory diseases, like COPD and chronic bronchitis. The sputum concentration of TNF- α have been found to be high in COPD patients[113], especially during exacerbations[114]. In serum, the level of TNF- α has been found to be increased in patients with severe COPD with cachexia[115]. However, in mild or moderate COPD, levels of TNF- α in saliva and serum as well as BAL macrophage TNF- α mRNA expression were found to be slightly attenuated[7].

Interleukin-8 (CXCL8/IL-8) is an abundant and well-studied chemokine. It is not stored intracellularly and it is produced by various cell types like macrophages, epithelial cells and endothelial cells *etc.*[116]. The main function of CXCL8 is to work as a potent chemoattractant to recruit neutrophils. Also, CXCL8 is responsible for activation and migration of other immune cells to inflammatory sites[117], for inducing cell phagocytosis[118], initiating respiratory burst[119] and promoting angiogenesis[120]. The effects of CXCL8 are triggered by binding to CXCR1 (low affinity receptor and displayed a relative selectivity for CXCL8) and CXCR2 (high affinity receptors and shared by many chemokines)[104]. As a neutrophilic chemotactic factor, it is not surprising that CXCL8 are important during the development of lung diseases like COPD. Compared with health controls the levels of CXCL8 have been found to be significantly higher in induced sputum[7] and BAL fluid[121] in COPD patients with further increase during exacerbations[114]. A negative relationship between saliva levels of CXCL8 and lung function index was also found in COPD patients[7]. Airway epithelial cells and alveolar macrophages from COPD patients secreted more CXCL8 than cells from smokers without COPD[122, 123].

Matrix metalloproteinases (MMPs) is a family of zinc-dependent proteinase. It is mainly produced by monocytes/macrophages, but neutrophils, eosinophils and some of the structural cells like epithelial cells are also known cell sources[124, 125]. MMPs play key roles in causing extensive alterations of lung extracellular matrix (ECM) as they are able to cleave structural proteins[126]. Matrix metalloproteinases-9 (MMP-9) is expressed at low levels in healthy lung but overexpressed in many lung diseases[126]. It is involved in lung epithelial wound repair by remodeling the provisional ECM and control the migration of repairing cells[127]. The activity of MMP-9 is usually regulated by tissue inhibitor of metalloproteinases-1 (TIMP-1), and the imbalance between MMP-9 and TIMP-1 might be associated with lung tissue degradation leading to airflow obstruction[128]. Previous studies have shown that MMP-9 and MMP-

9/TIMP-1 levels were elevated in induced sputum[128], BAL fluid[129] and serum[7, 130] in COPD patients compared to healthy controls. Interestingly, one of our studies identified a negative relationship between salivary MMP-9 and lung function index (FEV₁)[7].

Interleukin-13 (IL-13) is a T helper type 2 (Th2) cells cytokine, which also can be synthesized by other cells like CD4 cells, mast cells, NK cells, basophils *etc.*[131]. Both *in vitro* (bronchial epithelial cells culture) and animal studies have demonstrated that, IL-13 induces mucus producing cell hyperplasia and stimulate the secretion of mucin via epidermal growth factor receptor (EGFR) activation[132, 133]. Mucus hypersecretion is a clinical hallmark of chronic bronchitis, and smokers with chronic bronchitis have more IL-13 expressing cells in bronchial mucosa than smokers without chronic bronchitis[134]. The IL-13 also decreases mRNA expression of cilia-related markers[135] and attenuates the epithelial cell capacity of maintaining its barrier function and/ or wounds repair[136].

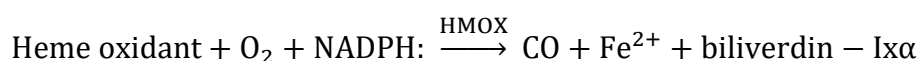
1.7 OXIDATIVE STRESS

Oxidative stress is a condition caused by an imbalance between oxidants and anti-oxidants leading to increasing levels of intracellular reactive oxygen species (ROS)[137]. ROS consists of superoxide anion (O²⁻), hydrogen peroxide (H₂O₂), and hydroxyl radicals (OH[•]) and are generated from both endogenous (normal cellular metabolism) and exogenous (exposure to environmental factors like air pollutants, irradiation or cigarette smoke) sources. High levels of ROS lead to cell damage including disruption of cell protein function and alternate DNA expression[138]. Therefore, ROS plays fundamental roles in cell viability, proliferation and organ function, as well as contributing to the pathogenesis of many chronic inflammatory airway diseases, such as COPD and asthma[139].

The survival of cells against oxidants is dependent on the intracellular redox homeostasis, which is maintained by the balance of ROS generation and elimination (conducted by anti-oxidant systems)[140]. After being activated by oxidative signals, transcription factor, NF-E2 related factor 2 (Nrf2), will leave Nrf2-Keap1 complexes which translocate into the nucleus. By binding to the anti-oxidant response element (ARE) in the promoter region[141], it will increase the anti-oxidant gene transcription potential (Figure 2 right). The main functions of anti-oxidants are scavenge superoxide and free radicals, and subsequently detoxify free radicals [142]. The major class of anti-oxidant enzymes includes heme oxygenase-1 (HMOX-1), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalases and glutathione S-transferases (GSTs). Besides, ROS can regulate the activity of NF-κB and further moderate the inflammatory gene expression by various pathways. For instance, ROS can directly or

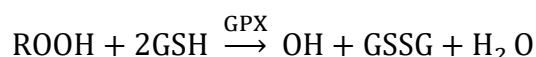
indirectly (by activate and inactivate the IKK complex) influence phosphorylation of I κ B α [143]. Also, ROS can alternate the DNA binding properties of the NF- κ B proteins themselves[144].

Heme oxygenase (HMOX) can catalyze the oxidative degradation of heme by interaction with molecular oxygen and cytochrome p450 reductase, resulting in the generation of carbon monoxide (CO), iron and biliverdin-Ix α [145]. Actually, HMOX also exerts anti-oxidative, anti-inflammatory and anti-proliferative properties via the products formed during these reactions. As examples the ability of CO to inhibit cell proliferation[146] and the sequestration of iron by ferritin with accompanying antioxidant[147] and anti-apoptotic[148] effects could be mentioned. Biliverdin-Ix α will eventually be reduced to bilirubin-Ix α by biliverdin reductase, and bilirubin-Ix α can function as antioxidant and anti-inflammatory mediator[149].



Two HMOX isoforms are known in humans, an inducible isoform (HMOX-1) and a constitutive isoform (HMOX-2). During hyperoxia, HMOX-1 is a strongly and highly regulated enzyme, and induced by a variety of lung cells, like alveolar macrophages, epithelial cells and fibroblasts[150]. It has been reported, that HMOX-1 expression was associated with COPD pathogenesis[145]. Level of HMOX-1 was increased in the alveolar spaces of smokers with and without COPD compared to non-smokers[151]. In alveolar macrophage, HMOX-1 expression was lower[152] while numbers of HMOX-1⁺ alveolar macrophage was higher[151] in smokers with COPD than non-smokers.

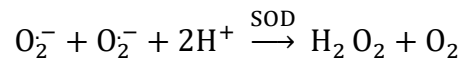
GPx usual bind to the selenocysteine (Sec) and converts peroxides, hydroxyl radicals or lipid hydroperoxides into water and alcohol[153, 154]. This process often concomitants with the oxidation of reduced glutathione (GSH) into oxidized glutathione (GSSG)[155]. With the help of glutathione reductase, GSSG can be recycled to GSH, which can keep intracellular GSH in an appropriate level[156].



There are 8 GPx isoforms (GPx-1-8) in humans, with GPx-1 as the most abundant isozyme which localized in the cytoplasm and can be found in nearly all tissues[154]. In respiratory system, there is a high amount of GPx in alveolar epithelial lining fluid, also cells like primary bronchial epithelial cells or alveolar macrophages can synthesize and secrete GPx[157]. It has been proved that GPx activity is associated with lung function impairment in COPD, assessed as decreased FEV₁[158]. According to recent studies[159, 160], GPx activity was significantly

reduced in smokers and COPD patients, and the reduction was correlated to the COPD severity based on GOLD criteria.

SOD catalyzes the highly reactive radical superoxide into less reactive radical hydrogen peroxide and molecular oxygen[142]:



In humans, 3 forms of SOD have been identified: cytosolic SOD (CuZn-SOD, SOD1), mitochondrial SOD (Mn-SOD, SOD2), and extracellular SOD (EC-SOD, SOD3)[161]. SOD3 which is located in the extracellular matrix (ECM) is highly expressed in lungs and vessels[162]. The SOD3 variants have been proved to relate to lung function decline in COPD patients[163]. Also the reduction of SOD3 level was found in COPD patients[164]. As SOD3 affect lung matrix homeostasis[165] it may offer protection against pulmonary disease.

2 AIMS OF THE STUDY

To elucidate the regulation of Toll-like receptor (TLR) in alveolar macrophages from healthy controls, smokers with and without chronic obstructive pulmonary disease (COPD), following stimulation with TLR ligands in combination with a glucocorticosteroid.

To develop a system which combine human 3D cell models, including primary bronchial epithelial cells (PBEC) and fibroblasts cultured at air-liquid interface (ALI) using the Xpose*ALI* exposure module.

To evaluate inflammatory and/ or oxidative stress effects of palladium and carbon nanoparticles exposure in both normal and chronic bronchitis-like lung 3D models.

To further elucidate the cell-cell interactions between PBEC in PBEC-ALI models and macrophages upon diesel exhaust particle exposure, regarding MQ polarization, inflammatory and oxidative stress responses.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Cells

3.1.1.1 Alveolar macrophages (AMQ)

The AMQ were isolated from 10 non-allergic, non-asthmatic, non-smoking healthy controls, 11 smokers without and 10 smokers with COPD. Detailed characterizations of the subjects and performance of bronchoscopy and BAL fluid has previously been described[7]. Cells from BAL fluid were re-suspended in 1 ml RPMI-1640 cell medium (Life technologies, Paisley, UK) supplemented with L-glutamine (1 %; Life technologies, Paisley, UK), penicillin streptomycin antibiotics (PEST, 1 %; Lonza, Basel, Switzerland) and heat-inactivated FBS (5 %; Life technologies, Paisley, UK) and seeded into 6 well plates (0.5 million cells/well). After 2 hours, macrophage that had adhered were cultured in 2 ml serum-free RPMI cell culture medium in 5% CO₂ at 37°C for 20 hours before treatment (for more details see Paper I).

3.1.1.2 Human primary bronchial epithelial cells (PBEC)

The PBEC were harvested from healthy bronchial tissues obtained from 10 different donors in connection with lung surgery (lobectomy). Such cells have been used in earlier studies[52, 166] and are well characterized. The PBEC were cultured in Keratinocyte serum-free medium (KSFM; Life technologies, Paisley, UK.) supplemented with human recombinant epidermal growth factor (EGF; 5 ng/ml; Life technologies, Paisley, UK), bovine pituitary extract (BPE; 50 µg/ml; Life technologies, Paisley, UK), PEST (1 %) and retinoic acid (RA; 10⁻⁵M; Sigma, Germany) and maintained in 5% CO₂ at 37°C (Paper II). In Paper III and IV, for the submerged culturing, PneumaCult™-Ex expand medium (Stemcell technologies, Cambridge, UK) supplemented with hydrocortisone (96 µg/ml, Stemcell technologies, Cambridge, UK) and PEST (1%) was used. Culture medium was replaced every second day. The cells were used at passage 3 with cells from at least 3 different donors (Paper II, III and IV).

3.1.1.3 Human fibroblast cell line (MRC-5)

The MRC-5 is a human fetal lung fibroblast cell line (American Type Culture Collection, ATCC, Rockville, MD, USA), frozen at passage 24. MRC-5 cells were cultured in Dulbecco's modified eagle medium (DMEM; Life technologies, Paisley, UK) supplemented with HEPES (1 %; Life technologies, Paisley, UK), non-essential amino acids (1 %; Sigma, Germany), PEST (1 %) and heat-inactivated FBS (10 %) and maintained in 5% CO₂ at 37°C. Culture medium was replaced every second day. The cells were used at passage 26 (Paper II).

3.1.1.4 Human monocytic cell line (THP-1)

The THP-1 is a human monocytic cell line derived from human blood (TIB-202™, ACTT). THP-1 cells were cultured in RPMI-1640 cell medium (Life technologies, Paisley, UK) supplemented with PEST (1 %) and heat-inactivated FBS (10 %) and maintained in 5% CO₂ at 37°C. Culture medium was replaced every second day. When the cell concentration reached 8 x 10⁵ cells/mL, they were sub-cultured at a concentration of 1 x 10⁶ cells/ml (for more details see Paper IV).

3.1.2 Particles generation

3.1.2.1 Pd nanoparticles (Pd-NP)

The Pd-NP was prepared by solvothermal technique with a modified procedure[167]. The formed particles were separated by centrifugation, washed by 99.5 % ethanol and dried in vacuum (for more details see Paper II).

3.1.2.2 Carbon Nanoparticles (CNP)

Endotoxin free Printex 90 particles were collected in Degussa (Frankfurt, Germany) and was a kind gift from Dr. Tobias Stöger's lab (Helmholtz Centrum, Munich, Germany)[168]. The printex 90 particles were suspended in pyrogen free distilled water at a concentration of 20 µg/50 µl (for more details see Paper III).

3.1.2.3 Diesel exhaust particles (DEP)

The DEP were generated from a three-cylinder tractor engine (Model 1113 TR; Bolinder-Munktel) ran on diesel fuel (Swedish environment class MK 3). The exhaust, was precipitated and scraped from a Tepcon electrostatic filter (Model 2200; ActAir, Cardiff, UK) and stored in the dark at -20°C (Paper IV).

3.1.3 Particle characterization

3.1.3.1 Pd-NP

Scanning electron microscope (SEM), Energy dispersive spectroscopy (EDS) (Hitachi TM 1000-µ-DeX) and X-ray powder diffraction (multifunctional Bruker SMART Apex-II diffractometer) were performed to ensure Pd-NP purity and crystallinity. The individual and hydrodynamic (ethanol/KSFM medium) size of Pd-NP were determined by image analysis in transmission electron microscope (TEM) and Laser reflection microscope (Malvern Nanosight instrument)[167] respectively (for more detail see Paper II).

3.1.3.2 CNP

Zetatrac (Model NPA151--31A; Particle Metrix GmbH, Meerbusch, Germany) was performed to detect the zeta potential and intensity weighted median dynamic light scattering diameter of Printex 90 particles (for more details see Paper III)

3.1.3.3 DEP

The 9-stage Marple Cascade Impactor (MCI, MSP Corporation, Minneapolis, USA) together with PreciseInhale™ platform (Inhalation Sciences Sweden AB, Stockholm, Sweden) were performed to analysis size distribution of DEP. (for more details see Paper IV)

3.1.3.4 Endotoxin Analysis

Limulus amebocyte lysate (LAL) assay was used to exclude the contamination of LPS in Pd-NP, CNs and DEP. The assay was performed according to the manufacture instructions (Endosafe®, Charleston, SC, USA). (for more details see Paper II, III and IV)

3.2 METHODS

3.2.1 Stimulation of alveolar macrophages (AMQ)

The AMQ were exposed to LPS (1µg/ml, *Escherichia coli* 0111: B4; Sigma, Germany), PGN (1µg/ml, Sigma, Germany) or TNF-α (10ng/ml; R&D SYSTEMS®, Europe, Abingdon, UK) with and without budesonide (Bud) (10⁻⁸ M, Sigma, Sweden). The cells were incubated in 5% CO₂ at 37 °C for 6 hours. Culture medium was collected and kept in -80°C for further analysis. Total mRNA of AMQ was isolated (4 from healthy controls and 5 from each smoking groups). (Paper I)

Monoclonal antibody (mAb) was added to block TLR2 or TLR4 (TLR2, TL2.1; TLR4, HTA 125; eBioscience, San Diego, CA, USA), and IgG2a was used as isotype control. (Paper I)

3.2.2 Bronchial mucosa model (3D model) establishment

3.2.2.1 Normal 3D model

The whole procedure took around 30 days (Table 2)[169] including cell expansion, establishment of the models and airlifting the models.

Table 2. Timeline and different procedures involved in establishing the model[169].

	Day1-7	Day8-14	Day15	Day16-30
PBEC expansion				
MRC-5 expansion				
Seed PBEC on inserts				
Culture in submersion				
Add MRC-5 on other side of inserts				
Culture in submersion				
Airlift the model				
Add IL-13 in airlifted medium of modified model				

Figure 4[169] illustrates the main steps in establishing our models. To set up the models, PBEC were seeded at a concentration of 1×10^5 cell/cm² and cultured on transwell inserts (0.4 μ M pore size, BD Falcon™) in twelve-well plate. Complete KSFM medium (with all supplements) was added to both basal and apical side of the insert and maintained in 5 % CO₂ at 37°C. Culture medium was changed every second day. After 1 week, the number of cells reached around 3×10^5 cells/cm². Then 1×10^4 cells/ml of fibroblasts were added to the downside of the insert membrane. The models were airlifted by adding airlifted medium (complete KSFM medium supplemented with CaCl₂ in ddH₂O (6 μ g/ml; Sigma, Germany), ethanolamine in ddH₂O (15 ng/ml; Sigma, Germany) and RA (10^{-5} M)) only in the basolateral chamber to let the apical chamber be exposed to the air. The models were maintained in 5 % CO₂ at 37 °C and airlifted medium was changed every second day. (for more details see Paper II)

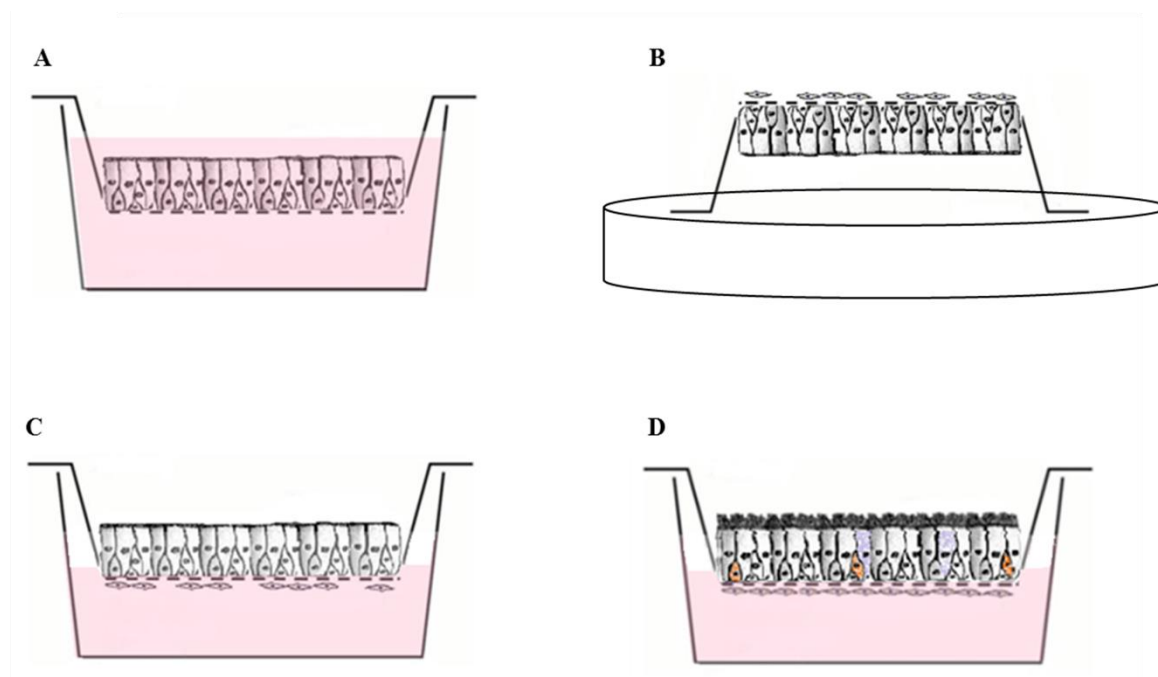


Figure 4. A diagrammatic representation of the main steps in establishing the model.

A: Apical seeding of PBEC into a 0.4 μ m semiporous transwell insert; B: Basolateral seeding of fibroblast; C: Removed medium and only added airlifted medium in the basal chamber; D: Differentiation when culturing at ALI. All steps were performed under sterile conditions and cells were cultured in 5 % CO₂ at 37 °C.

3.2.2.2 *Chronic bronchitis-like 3D model*

In order to develop chronic bronchitis-like 3D models, 1 ng/ml or 10 ng/ml human IL-13 (R&D SYSTEMS®, UK) was added together with the airlifted medium under ALI condition. All the other procedures were the same as normal 3D model. (for more details see Paper II and III)

3.2.3 **Bronchial mucosa model (3D model) characterization**

3.2.3.1 *Histological and immunofluorescence analysis*

For histological analysis, the model membranes were cut from insert, fixed, dehydrated, paraffin embedded and sectioned. The sections were stained with Hematoxylin and Eosin (H&E) or Periodic acid–Schiff (PAS) and captured using BX50 light microscope (Olympus Optical Co., Tokyo, Japan). (for more details see Paper II)

For immunofluorescence analysis, the fixed model membranes were stained with primary antibodies. Mouse anti-acetylated alpha tubulin antibody was used to identify ciliated cells and rabbit anti-mucin 5AC antibody to identify mucus producing cells (Abcam, Cambridge, UK). The staining was followed by the use of secondary antibodies, Alexa Fluor® 488-conjugated goat anti-mouse IgG and Alexa Fluor® 555-conjugated goat anti-rabbit IgG (Abcam, Cambridge, UK) and then mounted on microscope slides with DAPI (Abcam, Cambridge, UK). Negative control slides were also prepared by excluding the primary antibodies. Images were captured and visualized using a LSM700 confocal microscope (Zeiss, Germany). (for more details see Paper II)

3.2.3.2 *Scanning (SEM) and transmission (TEM) electron microscopy*

The membranes including models were cut from inserts, fixed and dehydrated. For SEM analysis, the dehydrated membranes were mounted and sputter-coated for examination. For TEM analysis, the dehydrated membranes were embedded in LX-112 (Ladd, Burlington, USA) and sectioned on an ultra-microtome. Before examination the sections were contrasted with uranyl acetate and followed by lead citrate. (for more details see Paper II)

3.2.3.3 *Transepithelial electrical resistance (TEER) measurement*

The TEER were measured with an EVOM voltage-ohm meter and chopstick electrodes (World Precision Instruments, New Haven, USA). $TEER = (TEER \text{ value detected} - TEER \text{ value of insert without cells}) \times 0.9$ (insert surface area). (for more details see Paper II)

3.2.3.4 Quantification analysis of specific markers

Real-time PCR

Fibroblasts were gently scraped from the models and the total mRNA of remaining PBEC was isolated by PureLink[®] RNA Mini Kit (Life technologies, Paisley, UK). Concentration of RNA was measured using the Nanodrop (ND1000 Technology). First-strand cDNA was synthesized with 1 µg mRNA using High-Capacity RNA-to-cDNA[™] Kit (Life technologies, Paisley, UK). Fast SYBR[®] Green Master Mix (Life technologies, Paisley, UK) was used to perform the RT-PCR with specific primers (forkhead box protein J1 (*FOXJ1*), *MUC5AC*, club cell protein (*CC10*) and Keratin 5 (*KRT5*)). Beta actin was adopted as an internal control gene. Data were analyzed using 7500 Software v.2.0.1 and results were calculated as $2^{-\Delta Ct}$ ($\Delta Ct = Ct(\text{gene of interest}) - Ct(\text{beta actin})$). (for more details see Paper II)

ELISA

Measurements of CXCL8 in culture medium from different models were performed using an in-house ELISA[170]. The detection limit was 12.5-6400 pg/ml. Levels of CC10 in culture medium from model were measured using a purchased DouSet ELISA uteroglobin kit (R&D SYSTEMS[®], UK). The detection limit was 31.2 pg/ml. For all the duplicated samples, an intra-assay variation <10 % was accepted. (Paper II)

3.2.4 Co-cultured models including both PBEC-ALI and THP-1 derived macrophage (MQ)

3.2.4.1 PBEC-ALI model

The PBEC-ALI model was built up the same way as 3D model which was described in the previous section (3.2.2) but without adding fibroblasts. After submerge culturing in PneumaCult[™]-Ex expand medium, the PBEC reached confluence. Then the PBEC model was air-lifted by aspirating all the PneumaCult[™]-Ex expand medium and adding PneumaCult[™]-ALI maintenance medium (Stemcell technologies, Cambridge, UK) supplemented with hydrocortisone (96 µg/ml), heparin (2 mg/ml Stemcell technologies, Cambridge, UK) and PEST (1%) to the basal chamber only. The model was kept in 5 % CO₂ at 37 °C and change PneumaCult[™]-ALI complete medium in the basal chamber every second day. (Paper III, IV)

As for chronic bronchitis-like PBEC-ALI model (PBEC-ALI/CB), 1 ng/ml human IL-13 (R&D SYSTEMS[®], UK) was added to the PneumaCult[™]-ALI medium under ALI condition. All the other procedures were the same as PBEC-ALI. (Paper III)

3.2.4.2 THP-1 derived macrophages (MQ)

To differentiate THP-1 cell to macrophage-like cells[171], 5 ng/ml phorbol myristate acetate (PMA) (Sigma, Germany) was added to the culture medium[172]. After 48 hours incubation, the adherent cells which were supposed to be MQ were trypsinized and collected for further analysis. Anti-CD 68-PE-Cy7 (BD Pharmingen, San Diego, CA, United States) was used as a marker to detect the purity of the MQ by flow cytometry (LSR Fortessa™, BD Bioscienc, United States). (for more details see Paper IV)

3.2.4.3 Co-culture of PBEC model and MQ

MQ on top of the epithelial cells (PBEC-ALI/MQ)

Two hundred µl THP-1 culture medium containing 1.5×10^5 MQ was added to the apical side of the PBEC-ALI model (MQ: PBEC = 1:10) and incubated in 5% CO₂ at 37°C for 2 hours. Before exposure, after MQ adherence, medium at the apical chamber was aspirated. (for more details see Paper IV)

MQ underneath the model (PBEC-ALI/MQ_{sub})

After exposure, PBEC-ALI models were transferred to 12-wells plate which were pre-cultured with 1.5×10^5 MQ in the well underneath the insert with exposed PBEC and the incubation continued in 5% CO₂ at 37°C for 6 hours and 24 hours (For details see supplement in Paper IV)

3.2.5 Exposure with XposeALI/ system

Small portions of dry particle powder (Pd-NP, CNP and DEP) were generated to an aerosolized form and forced into the holding chamber (300ml) by compressed air (100-140 bars) using XposeALI system (PreciseInhale™ exposure platform). The main flow rate of generated aerosol was 90 ml/min (Paper II) or 120 ml/min (Paper III, IV) and divided into three consecutive branch flows of 10 ml/min for each exposure of triplicate model inserts at the same time. Models were exposed for different time durations in proportion to the desired target dose. Corresponding to low, medium and high exposure doses, the exposure cycles took 20 seconds, 45 seconds, 3 minutes (Paper II), and 1 minutes, 2 minutes, 3 minutes (Paper III). and 15 seconds, 45 seconds, 3 minutes (Paper IV). As a control, sham exposures was performed with identical settings using normal air and a clean exposure system.

After aerosol exposure, the models were incubated in 5 % CO₂ at 37 °C for 6 hours (Paper III), 8 hours (Paper II) or 24 hours (Paper II, III, IV). The apical medium (AM) was obtained by lavage on apical side of the epithelial layers for 15 minutes at room temperature (Paper II, III).

Both AM (Paper II, III) and medium from the basal chamber of the insert (BM) (Paper II, III, IV) were collected and frozen at -80 °C until further use.

3.2.6 Particle exposure dose and uptake

3.2.6.1 Particle exposure dose

To determine Pd-NP exposure doses, the model membranes were digested by aqua regia and neutralized to pH=3 by ammonia after exposure. Analyses were carried out by inductively coupled plasma mass spectrometer (ICP-MS)[173] with an isotopically enriched ¹⁰⁸Pd as standard. (for details see Paper II).

As for CNP and DEP exposure doses, after particle exposure, the membranes including models were rinsed with 99% ethanol. Analysis were carried out by spectrophotometer technique (Cary 60 UV-Vis, Agilent Technologies, Palo Alto, CA, United States). The gradient diluted CNP or DEP in 99% ethanol were recognized as standard (Paper III and IV).

The actual exposure dose of Pd-NP, CNP and DEP in each insert (PI) was calculated using the following formula:

$$\text{Particle exposure dose} = \frac{\text{PI}}{\text{IS (insert surface area)}}$$

3.2.6.2 Particle exposure uptake

The localization of the particles in models were detected by TEM (section 3.2.3.2) after incubation for 2, 4, 8 and 24 hours post Pd-NP exposure. (for details see Paper II)

To identify the uptake of DEP by MQ, a commercial quadri-wave lateral shearing interferometry (QWLSI) (SID4Bio, Phasics SA, Saint Aubin, France) was directly plugged onto microscope (Labphot-2, Nikon FX-35DX) to detect distribution of mass across the models (Paper IV).

3.2.7 Assessment of cytotoxicity

3.2.7.1 Trypan blue assay

To determine viability of the cells, trypan blue was used to assess cell membrane integrity. For re-suspended cells or cells after trypsinization, 10 µl 0.4% trypan blue was mixed with 10µl cell suspension, and 200 cells were counted to calculate the viability (Paper I-IV). For the models, before and 8/24 hours after exposure, 200 µl 0.2% trypan blue was added to the apical

side of the insert. Four fields of the insert were randomly selected and in each field 200 cells were counted (Paper II, IV). Viability of more than 95% was acceptable.

3.2.7.2 *Lactate dehydrogenase (LDH) assay*

Cell viability was estimated based on the measurement of LDH which is a cytosolic enzyme released into cell culture medium when plasma membranes are damaged. After exposure 50 µl of BM were assayed for LDH activity following the manufacturer's protocol. Results were presented as % Cytotoxicity = $\frac{\text{Particle exposed LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}}$. (Paper III and IV)

3.2.7.3 *Alamar Blue Assay*

To confirm the cell viability further Alamar Blue[®] Assay which is based on the detection of metabolically active cells was performed. The assay utilizes changes of the fluorescence of resazurin (blue, non-fluorescent) to the reduced form resorufin (red, highly fluorescent). Four hundred µl of 10% Alamar Blue[®] solution was added to the apical side of the model and collected after 2 hours. The absorbance was measured using spectrophotometer at an emission of 570 nm with 600 nm as a reference wavelength. (Paper III)

3.2.7.4 *Apoptosis assay*

After 6/8/24 hours culturing post exposure, models were trypsinized and treated with annexin V-PE/7-AAD according to the manufacturer's instructions (BD Pharmingen, San Diego, CA, United States). Apoptotic cells were detected by collecting 2000 cells using flow cytometry (LSR Fortessa[™], BD Bioscienc, United States) (Paper II-IV).

3.2.8 Measurement of inflammatory, oxidative stress and MQ polarization markers

3.2.8.1 *Analysis of mRNA*

In Paper I, after stimulation, AMQ mRNA from 4 healthy controls and from 5 smokers with and without COPD group were isolated by PureLink[®] RNA Mini Kit (Life technologies, Paisley, UK). First-strand cDNA was synthesized with 0.5 µg mRNA using QuantiTect[®] Reverse Transcription Kit (Qiagen NV, Venlo, the Netherlands). Fast SYBR[®] Green Master Mix (Life technologies, Paisley, UK) was used to perform the RT-PCR with primers (*TLR2* and *TLR4*).

In Paper III and IV, after 6/24 hours culturing post exposure, models were lysed. Total mRNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The following steps were the same as described in section 3.2.3.4. Primers of genes involved in pro-inflammation

(*CXCL8*, *IL6* and *TNF α*), innate immunity (*TLR2* and *TLR4*), tissue injury/repair (*MMP9* and *TIMP1*), oxidative stress (*NFKB*, *HMOX1*, *GPx*, *SOD3* and *GSTA1*), and macrophage polarization (**M1**; *IL23*, *IL12*, **M2**; *IL4*, *IL10*, *IL13*, *MRC1*, *MRC2*, *RETNLA*) was used.

For all the analysis, beta actin was adopted as an internal control gene. The data were analyzed using 7500 Software v.2.0.1, and results were calculated as $2^{-\Delta Ct}$ ($\Delta Ct = Ct \text{ (gene of interest)} - Ct \text{ (beta actin)}$). (Paper I, III and IV).

3.2.8.2 Analysis of protein levels

Flow cytometry

For PBEC-ALI, the expression of TLR2 and TLR4 were detected directly using anti-TLR2 and anti-TLR4 monoclonal antibodies (anti-TLR2-APC, anti-TLR4-PE, BD Pharmingen, San Diego, CA, United States). For PBEC-ALI/MQ, cells were treated with CD68 monoclonal antibody (anti-CD 68-PE-Cy7; BD Pharmingen, San Diego, CA, United States) to distinguish between PBEC and MQ in co-cultured model. Then TLR2 and TLR4 expression were detected in PBEC (CD68⁻) and MQ (CD68⁺) respectively. For all the detection, unstained cells were used to gating the positive populations[174]. Analyses were performed using flow cytometry (LSR Fortessa™, BD Bioscienc, United States) and calculated as median fluorescence intensity (MFI). (Paper IV)

ELISA

Concentrations of IL-6 and CXCL-8 in AM/BM were measured using the in-house ELISA method described previously[170]. Commercially available antibodies (R&D SYSTEMS®, UK) were used. The detection range was 3-375 pg/ml for IL-6 and 12.5-6400 pg/ml for CXCL-8. MMP-9, TIMP-1, CC-10, TGF- β , IL-13 and IL-10 in AM/BM were measured using purchased DouSet ELISA Kit (R&D SYSTEMS®, UK). The detection limit was 31.2 pg/ml for all the DouSet ELISA. The measurements of TNF- α in BM were performed by purchased DouSet or HS quantikine ELISA Kit (R&D SYSTEMS®, UK). The detection limit was 7.8 pg/ml for DouSet and 0.049 pg/ml for HS quantikine ELISA. All the analyses were performed according to the manufacturer. For all the duplicated samples, an intra-assay variation <10 % was accepted (<20 % for TNF- α HS quantikine ELISA).

3.2.9 Statistics

Depending on the distribution of the data, the results are presented as mean values and 95 % confidence interval (95% CI) or median values and 25th–75th percentiles.

As the sample size was small in all studies, also the distribution was not clear for every outcome, non-parametric statistical methods were used in this thesis (except lung function index in Paper I). The comparison between groups, were performed by Kruskal–Wallis test followed by the Mann–Whitney U-test as a *post hoc* test (Paper I). The comparisons within groups (Paper I) or between different models (Paper II-IV) were assessed by Friedman test if more than 2 groups followed by Wilcoxon signed rank t test as a *post hoc* test or Wilcoxon signed rank test direct if only 2 groups. A p-value <0.05 was considered as significant. All the data were analyzed using STATISTICA9 (StatSoft, Inc. Uppsala, Sweden).

4 RESULTS

4.1 PAPER I

In Paper I, the aim was to explore whether TLR ligands alter Toll-like receptor expression (TLR2, TLR4) and to what extent a glucocorticosteroid interacts with this ligand-receptor interaction on immune effector cells.

The experiments were performed on alveolar macrophages (AMQ) isolated from BAL-fluid from healthy controls and smokers with and COPD. The AMQ were stimulated with peptidoglycan (PGN), lipopolysaccharide (LPS), or TNF- α with and without the addition of budesonide (Bud). The TLR2 or TLR4 blocking antibodies were also added before identical stimulations. The levels of secreted pro-inflammatory cytokines and chemokines as well as mRNA expression of *TLR2* and *TLR4* were measured after all different stimulations.

For further details, see paper I. All the figures have been published and permitted to be used here[175].

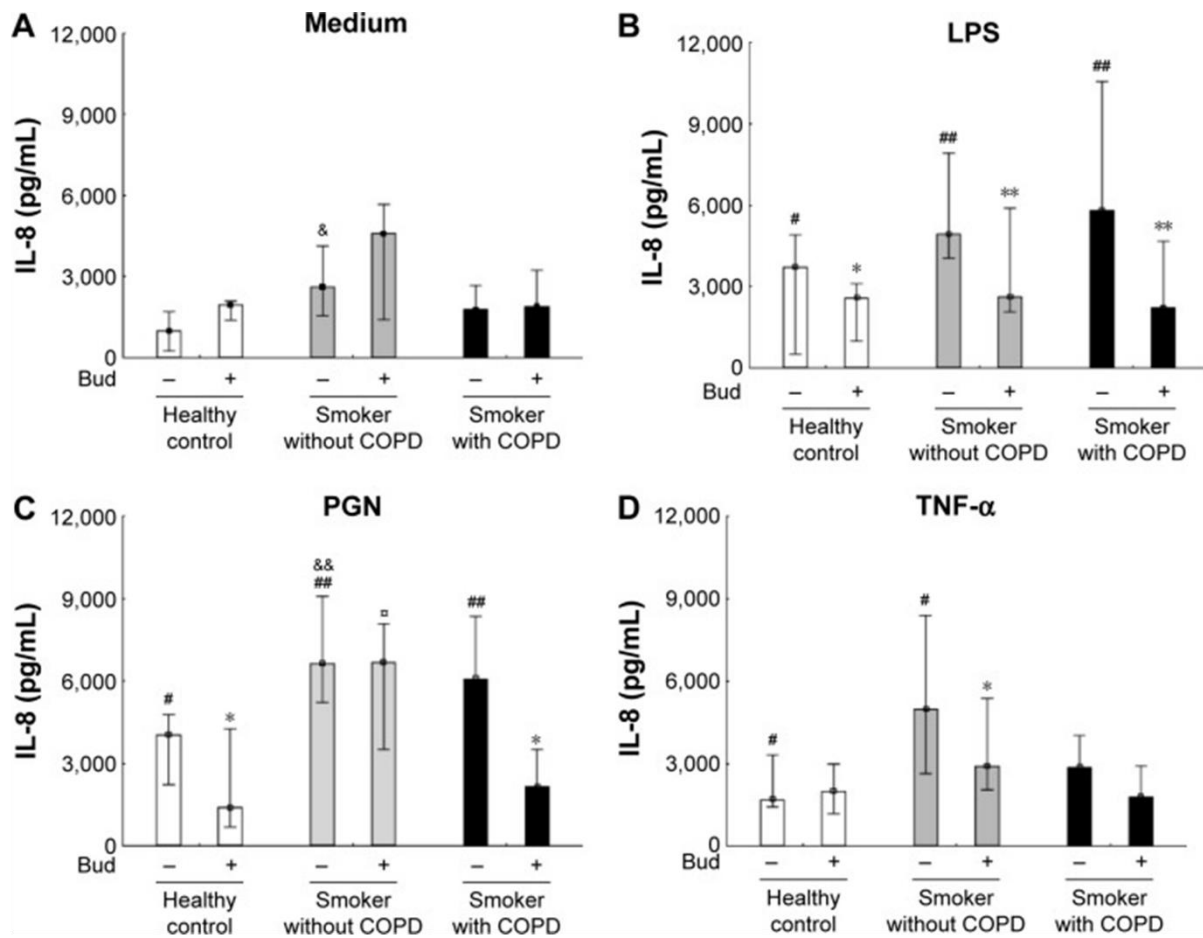


Figure 5. Secretion of IL-8/CXCL-8 in BAL fluid alveolar macrophages from the healthy controls and smokers with and without chronic obstructive pulmonary disease (COPD) and incubated for 6 hours in control medium (A), or after stimulation with LPS (B), PGN (C), and TNF- α (D) in the absence and presence of Bud.

Data are presented as median values and interquartile ranges. #, ##: $P < 0.05$, < 0.01 for the effect of different stimuli (medium vs LPS/PGN/TNF- α). *, **: $P < 0.05$, < 0.01 for the effect of Bud (LPS/PGN/TNF- α vs LPS + Bud/PGN + Bud/TNF- α + Bud). ∘: $P < 0.05$ for the effect of PGN (Bud vs PGN + Bud). &, &&: $P < 0.05$, < 0.01 compared with the healthy control cells. BAL, bronchoalveolar lavage; LPS, lipopolysaccharide; PGN, peptidoglycan; TNF- α , tumor necrosis factor- α ; Bud, budesonide.

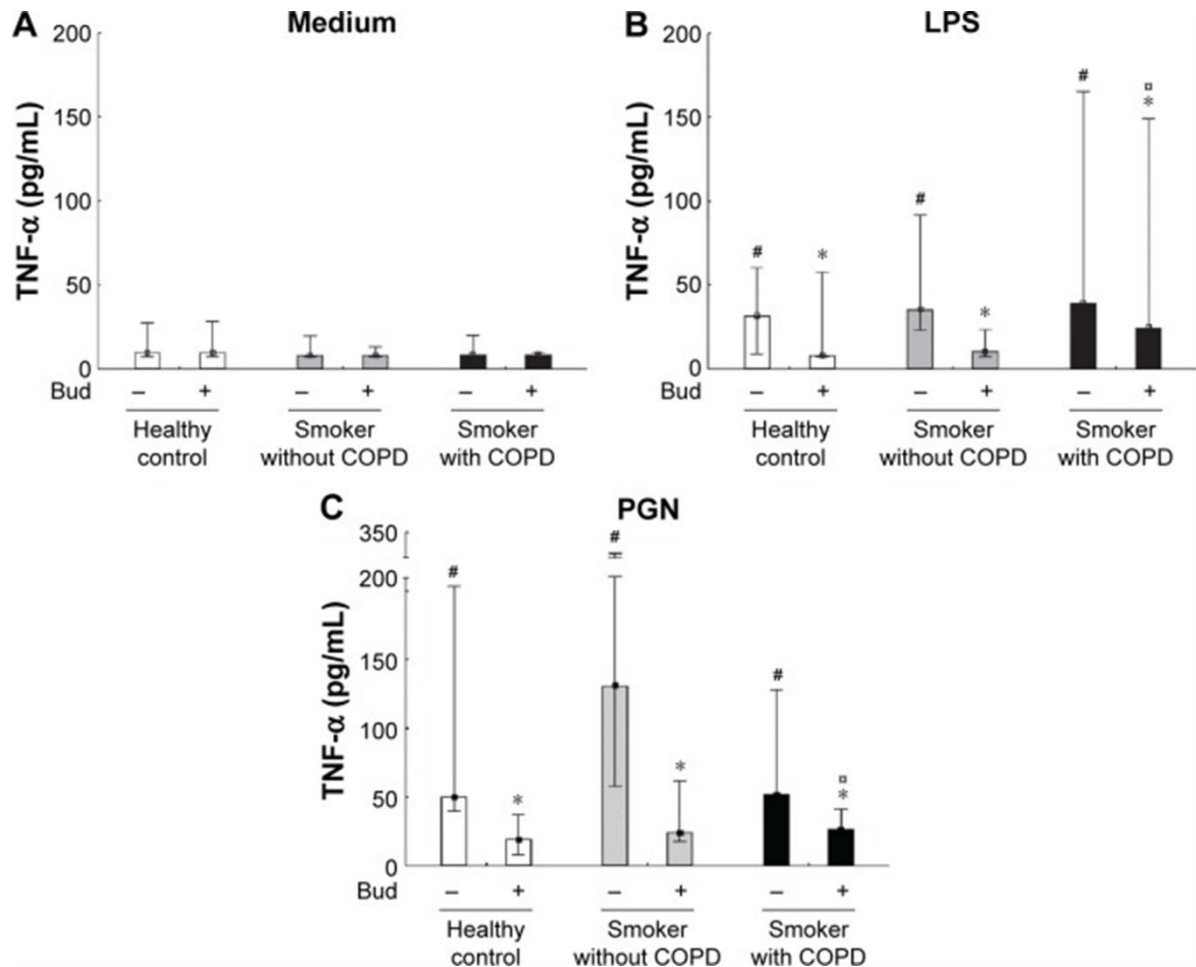


Figure 6. Secretion of TNF- α in BAL fluid alveolar macrophages from healthy controls and smokers with and without chronic obstructive pulmonary disease (COPD) and incubated for 6 hours in the control medium (A), or after stimulation with LPS (B), and PGN (C) in the absence and presence of Bud.

Data are presented as median values and interquartile ranges. #: $P < 0.05$ for the effect of different stimuli (medium vs LPS/PGN). *: $P < 0.05$ for the effect of Bud (LPS/PGN vs LPS + Bud/PGN + Bud). #: $P < 0.05$ for the effect of LPS/PGN (Bud vs LPS + Bud/PGN + Bud). TNF- α , tumor necrosis factor-alpha; BAL, bronchoalveolar lavage; LPS, lipopolysaccharide; PGN, peptidoglycan; Bud, budesonide.

In AMQ from all three groups LPS and PGN stimulation enhanced IL-8/CXCL-8 and TNF- α secretion which was attenuated by Bud (except PGN-induced IL-8/CXCL-8 release in smokers without COPD) (Figure 5B&C; Figure 6B&C). TNF- α increased the release of IL-8/CXCL-8 in AMQ from healthy controls and smokers without COPD which was inhibited by Bud in the latter group (Figure 5D).

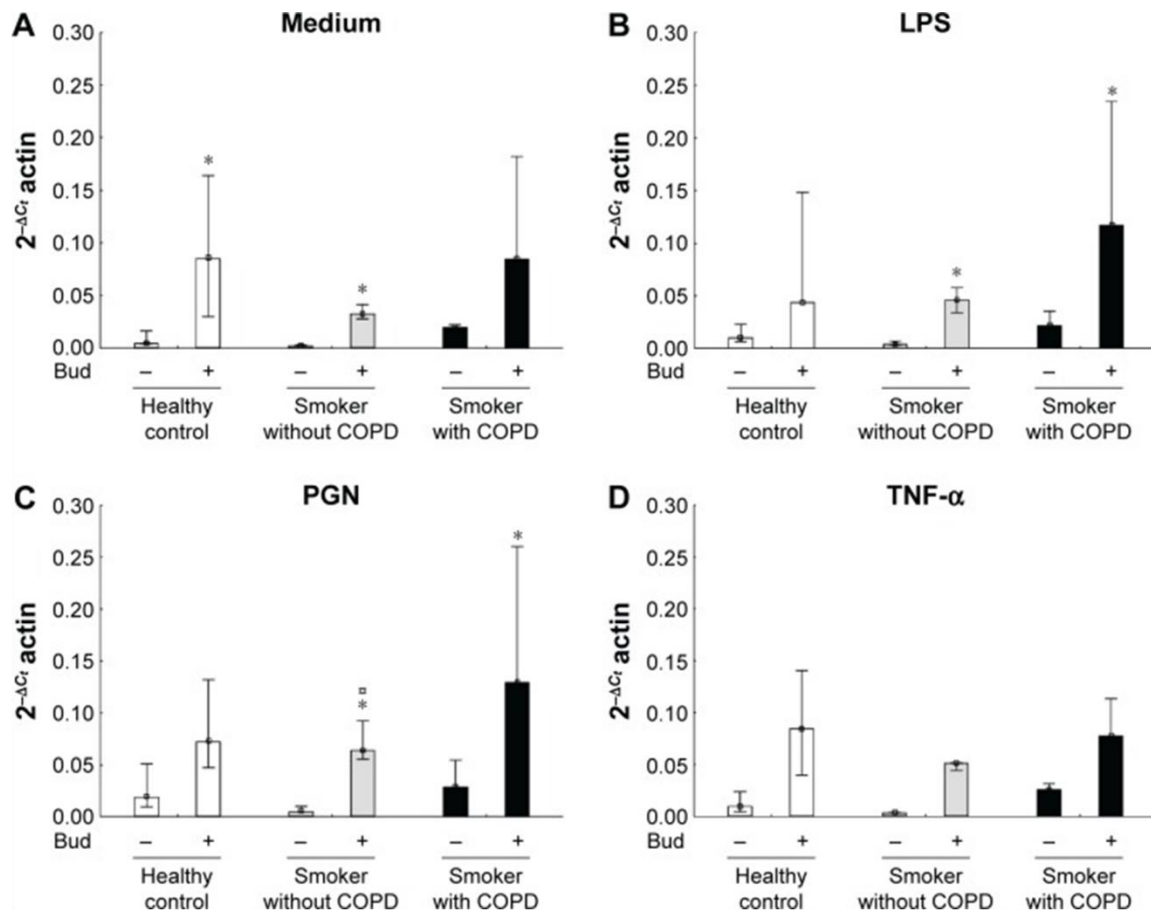


Figure 7. Expression of TLR2 mRNA in BAL fluid alveolar macrophages from the healthy controls and smokers with and without chronic obstructive pulmonary disease (COPD) and incubated for 6 hours in the control medium (A), or after stimulation with LPS (B), PGN (C), and TNF- α (D) in the absence and presence of Bud.

Data are presented as median values and interquartile ranges. *: $P < 0.05$ for the effect of Bud (medium/LPS/PGN vs Bud/LPS + Bud/PGN + Bud). **: $P < 0.05$ for the effect of PGN (Bud vs PGN + Bud). TLR2, toll-like receptor 2; BAL, bronchoalveolar lavage; LPS, lipopolysaccharide; PGN, peptidoglycan; TNF- α , tumor necrosis factor- α ; Bud, budesonide.

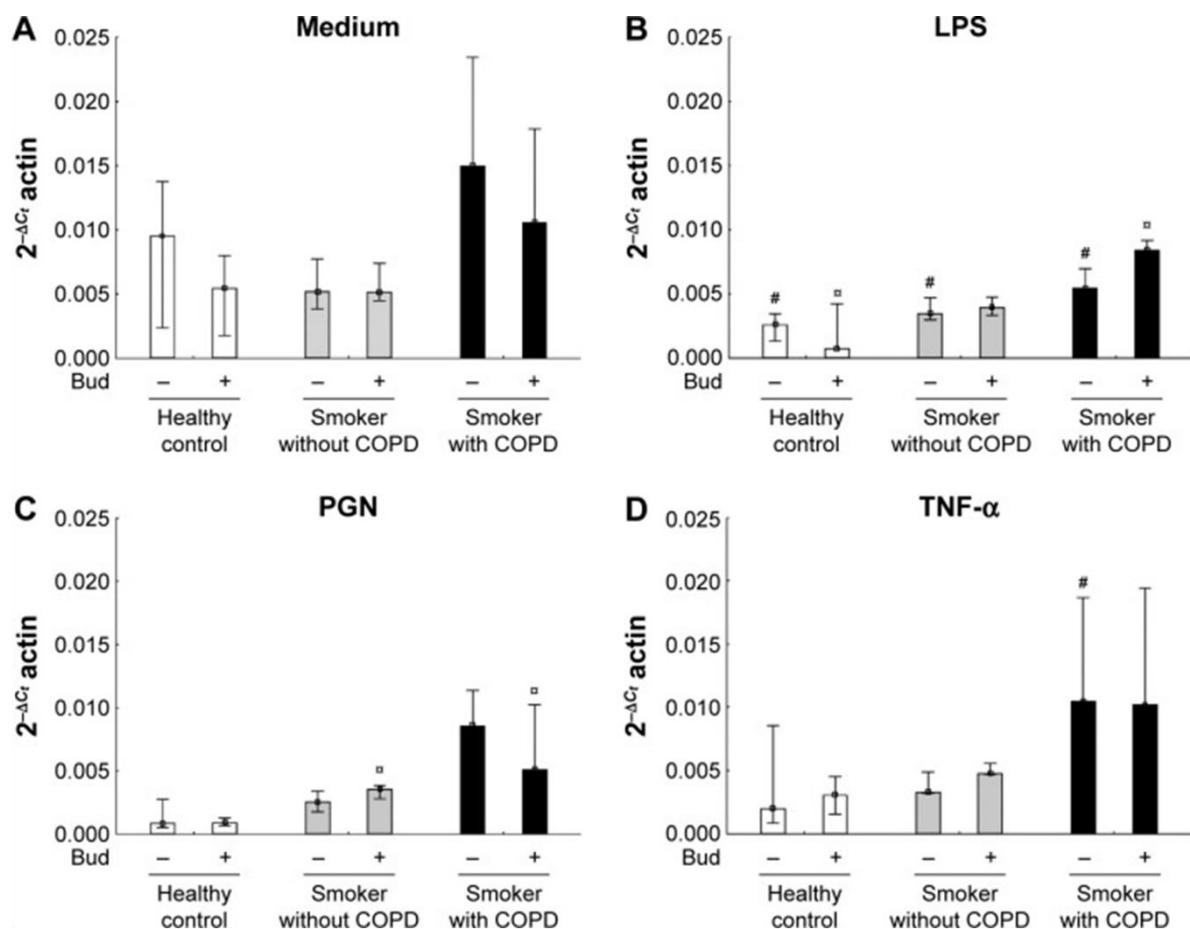


Figure 8. Expression of TLR4 mRNA in BAL fluid alveolar macrophages from the healthy controls and smokers with and without chronic obstructive pulmonary disease (COPD) and incubated for 6 hours in the control medium (A), or after stimulation with LPS (B), PGN (C) and TNF- α (D) in the absence and presence of Bud.

Data are presented as median values and interquartile ranges. #: $P < 0.05$ for the effect of different stimuli (medium vs LPS/TNF- α). □: $P < 0.05$ for the effect of LPS/PGN (Bud vs LPS + Bud/PGN + Bud). TLR4, toll-like receptor 4; BAL, bronchoalveolar lavage; LPS, lipopolysaccharide; TNF- α , tumor necrosis factor- α ; PGN, peptidoglycan; Bud, budesonide.

As for TLR mRNA expression, Bud itself increased *TLR2* mRNA expression in non-smokers and smokers without COPD (Figure 7A). The combination of Bud and TLR ligands (PGN or LPS) also increased *TLR2* mRNA expression in smokers with and without COPD (Figure 7B&C). Further, stimulation with LPS decreased the transcript expression of *TLR4* in all three groups (Figure 8B).

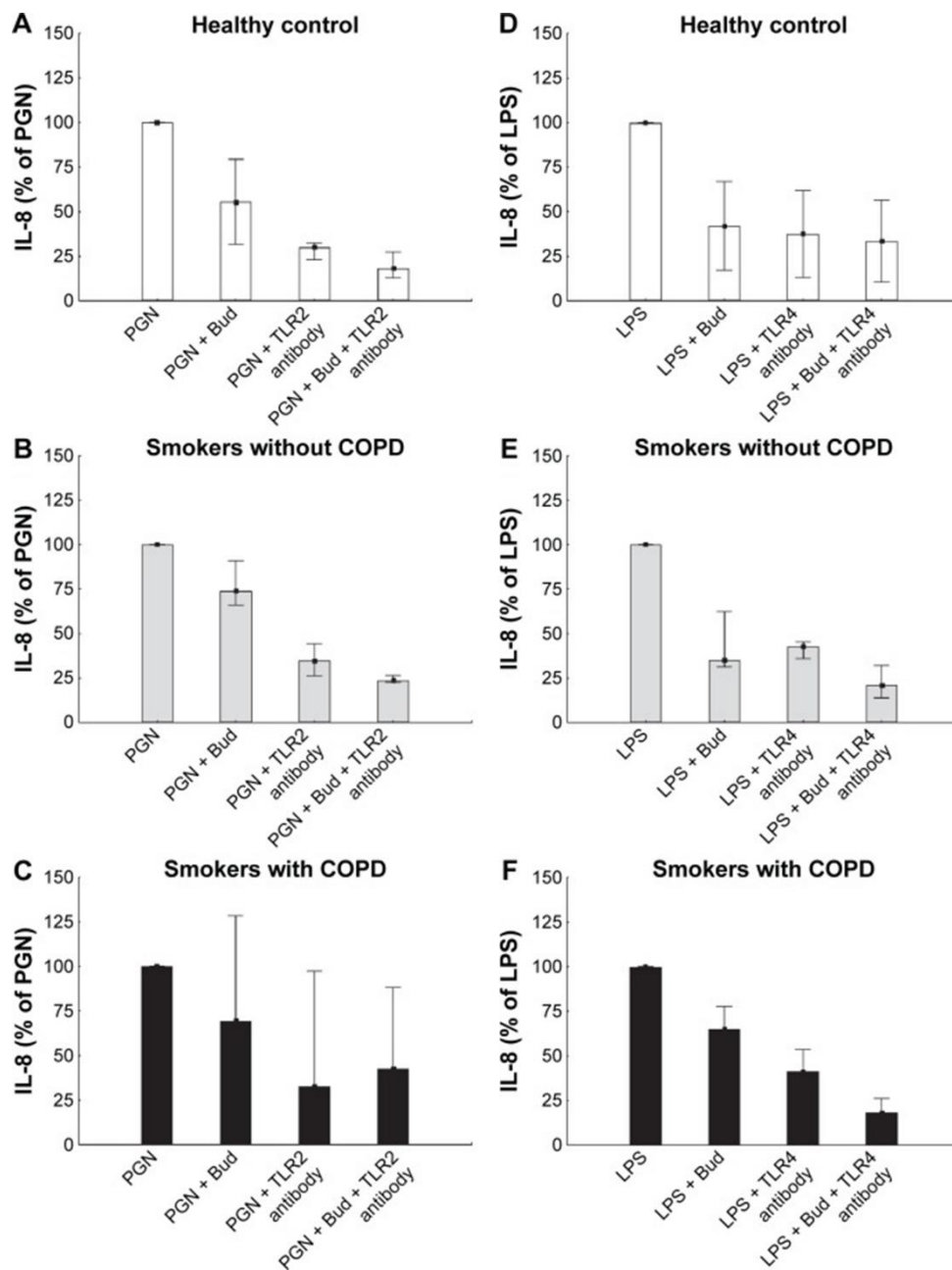


Figure 9. Inhibition of IL-8/CXCL-8 secretion in response to PGN (A–C) or LPS (D–F) stimulation by Bud after pre-incubation without or with TLR2/TLR4 antibodies from the healthy controls and smokers with and without chronic obstructive pulmonary disease (COPD).

Values are presented as percentage of IL-8/CXCL-8 secretion stimulated by PGN (A–C) or LPS (D–F). Data are presented as median values and interquartile ranges. PGN, peptidoglycan; LPS, lipopolysaccharide; TLR2, toll-like receptor 2; TLR4, toll-like receptor 4; Bud, budesonide.

To identify whether the inflammatory effects of a stimulus are driven by TLR pathways, TLR2 and TLR4 were blocked by corresponding antibodies. The TLR-blockade tended to inhibit PGN and LPS induced IL-8/CXCL-8 secretion in all three groups (Figure 9).

4.2 PAPER II

In Paper II, the aim was to develop an advanced system consist of 3D model and a sophisticated exposure platform to resembles an *in vivo* situation.

The 3D model was built by human primary bronchial epithelial cells (PBEC) and a fibroblast cell line co-cultured under air liquid interface (ALI). To validate the 3D model and to clarify how PBEC differentiate under ALI culture condition, light-, confocal microscopy, scanning-, and transmission electron microscopy, RT-PCR, ELISA and TEER were performed. Trypan blue and FACS were used to identify cell viability and apoptosis rate in the models. To build chronic bronchitis-like 3D model, 1ng/ml IL-13 was added at the start of air-lifting.

After establishment, both normal and chronic bronchitis-like 3D models were exposed to custom synthesized size-uniform palladium nanoparticles (Pd-NP) at three different concentrations with XposeALI system and then incubated for 8 and 24 hours. Exposure to normal air and clean system served as a control. Levels of IL-8/CXCL-8 and MMP-9 were detected from apical lavage medium (AM) and basal medium (BM).

For further details, see paper II. All the figures have been published and permitted to be used here[169].

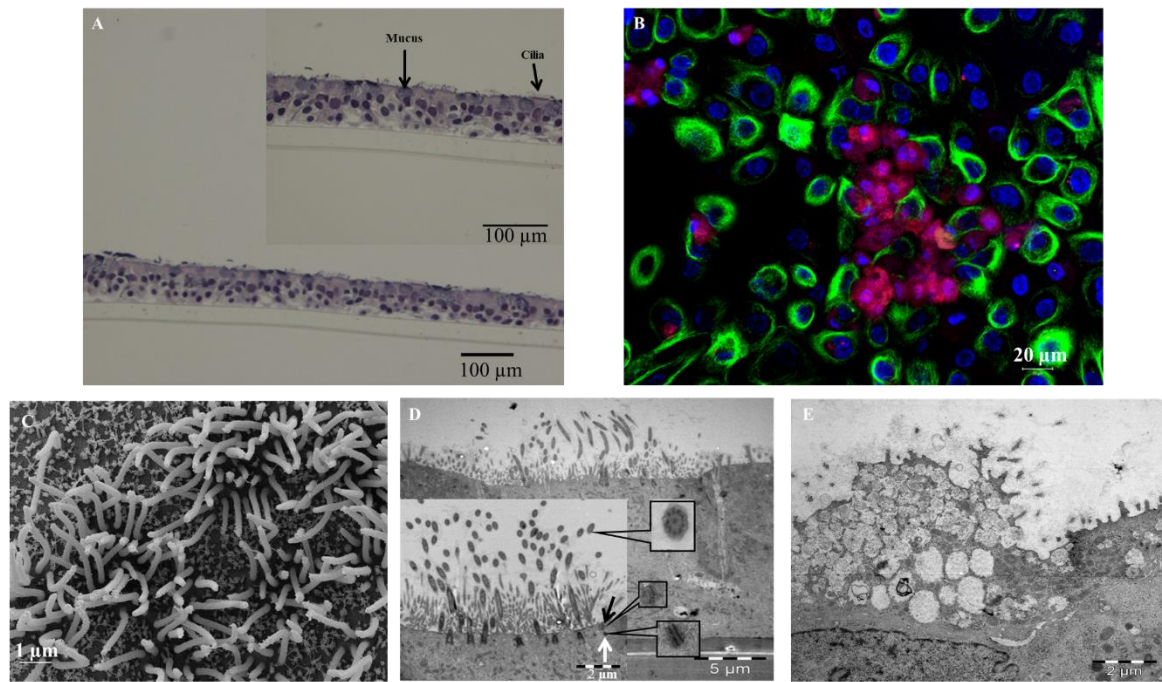


Figure 10. Morphological characterization of 3D model

A: Hematoxylin and Eosin (H&E) staining of paraffin embedded cross section of 2 weeks ALI model; Bar scale:100 μ m. Higher magnification showed cilia and mucus for a clearer view; Bar scale:100 μ m. B: Immunofluorescence staining of ciliated cell marker anti-acetylated alpha tubulin antibody (green fluorescence), mucus producing cell marker anti-MUC5AC antibody (red fluorescence) in 2 weeks ALI model; cell nuclei stained with DAPI (blue fluorescence). Bar scale:20 μ m. C: Scanning electron microscope analysis of 2 weeks ALI model, mature cilia present; Bar scale:1 μ m. D: Transmission electron microscope analysis of 2 weeks ALI model, ciliated cell surface was scattered with elongated cilia; Bar scale:5 μ m. Higher magnification showed cilia displaying 9+2 axoneme formation, tight junction (black arrow) and desmosome (white arrow); Bar scale:2 μ m. E: Transmission electron microscope analysis of 2 weeks ALI model, mucus cell present with electron-dense cytoplasm containing electron-lucent granules; Bar scale:2 μ m.

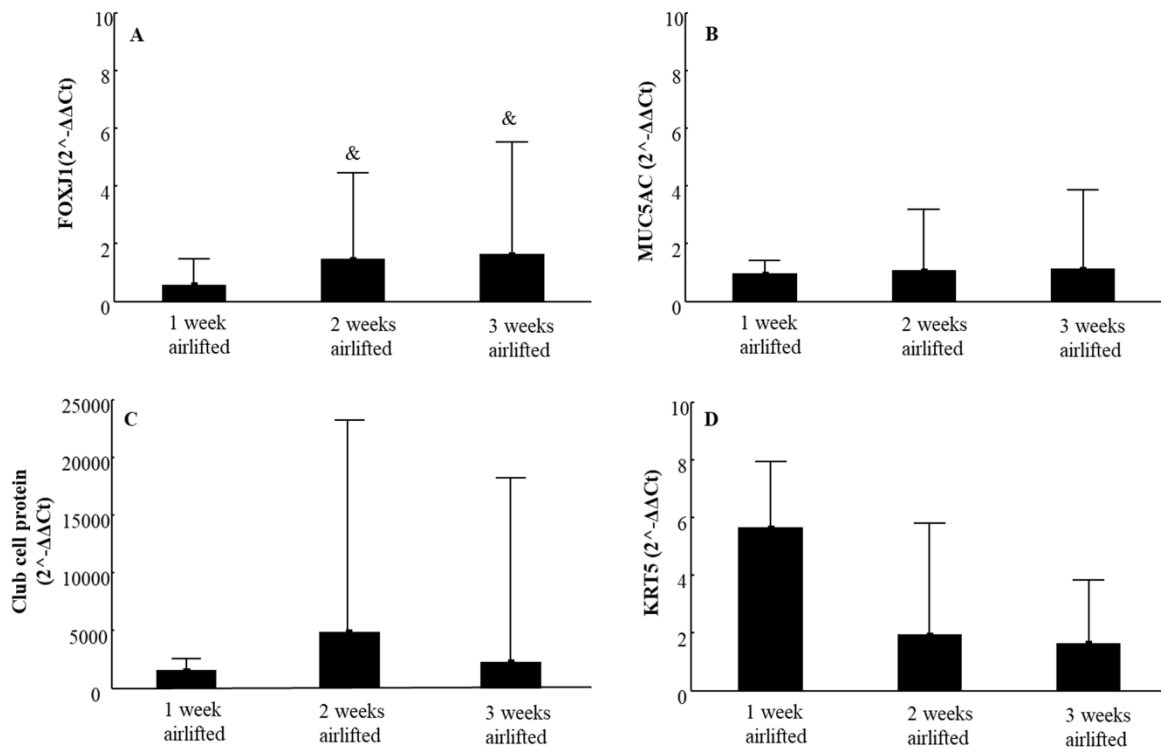


Figure 11. mRNA expression of different cell type markers in 3D models.

Expression of ciliated cell marker *FOXJ1* (A), mucus producing cell marker *MUC5AC* (B), club cell marker Club cell protein (C) and basal cell marker *KRT5* (D) mRNA in normal models after airlifted for 1, 2 and 3 weeks (N=9). Data present as median and 25th -75th percentiles; &: P<0.05 VS *FOXJ1* mRNA expression in 1 week airlifted models.

According to hematoxylin and eosin (H&E) staining analyses of cross sections of normal 3D model (Figure 10A), we found that PBEC build up a pseudo-stratified cell layer on the insert membrane and differentiate into basal cells, club cells, mucus producing cells and ciliated cells. Immunofluorescence analysis (Figure 10B), confirmed that 3D model contained ciliated cells which were covered by multiple cilia (green florescence) as well as mucus producing cells which can be stained with MUC5AC antibody (red florescence). The SEM (Figure 10C) and TEM (Figure 10D&E) analyses were performed to identify appearance and ultra-structure architecture of PBEC in 3D model. Ciliated cell surface was scattered with elongated cilia which showed axonemes consisting of two central singlet and nine outer doublet microtubules. Mucus producing cell showed an electron-dense cytoplasm that contained electron-lucent granules.

The mRNA expressions of different cell type specific markers (*FOXJ1* indicated ciliated cells, *MUC5AC* indicated mucus producing cells, Club cell protein indicated club cells and *KRT5* indicated basal cells) with different culture time points were detected by RT-PCR (Figure 11). From the results, the *FOXJ1* mRNA expression was significantly higher after being airlifted for 2 or 3 weeks compared to 1 week. However, there is no significantly changes for mRNA expressions of *MUC5AC*, Club cell protein and *KRT5* over time.

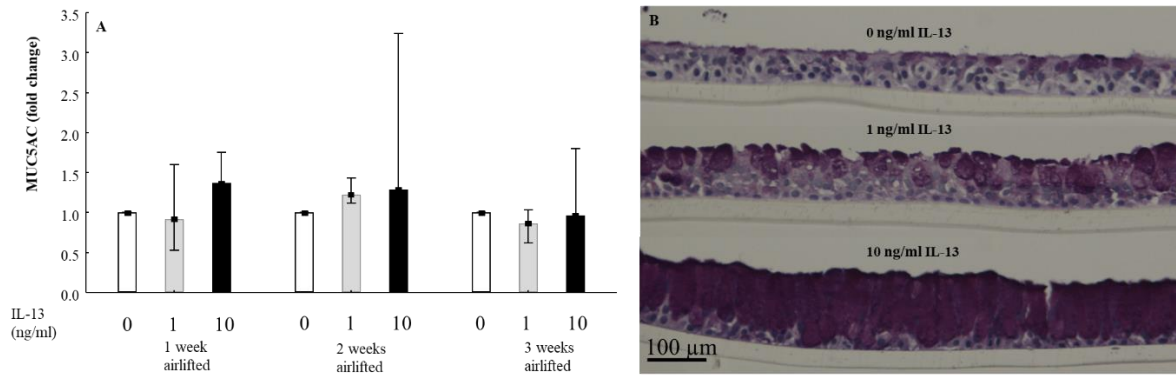


Figure 12. Effects of IL-13 stimulation on 3D models.

A: Fold change of *MUC5AC* mRNA expression in ALI models treated without (blank) and with 1 ng/ml (grey) and 10 ng/ml (black) IL-13 for 1, 2 and 3 weeks (N=9); Data present as median and 25th -75th percentiles. B: Periodic acid-Schiff (PAS) staining of paraffin embedded cross section of ALI models treated without and with 1ng/ml and 10ng/ml IL-13 for 2 weeks; Bar scale:100 μ m.

Treating airway epithelial cell with IL-13 induces mucus producing cell metaplasia and hyperplasia as well as increased mRNA expression of *MUC5AC*[132]. Therefore, different concentrations of IL-13 were tested to build up chronic bronchitis-like 3D model. We found that stimulation of the model with IL-13 increased *MUC5AC* mRNA expression (Figure 12A). Based on PAS stained cross sections (Figure 12B), model treated with IL-13 (1 ng/ml and 10 ng/ml) have more mucus producing cells than untreated model. However, with 10 ng/ml IL-13 treatment, almost all cells in the model produced mucus, which does not represent the real situation *in vivo*. Therefore, we used 1 ng/ml IL-13 to build chronic bronchitis-like 3D model. After treatment, the model contained different types of cell with more mucus producing cells.

The TEER reached $192 \pm 37 \Omega \cdot \text{cm}^2$ and $187 \pm 55 \Omega \cdot \text{cm}^2$ in the normal and chronic bronchitis-like 3D models respectively, with no significant differences in barrier integrity between normal and chronic bronchitis-like 3D models (Data not shown).

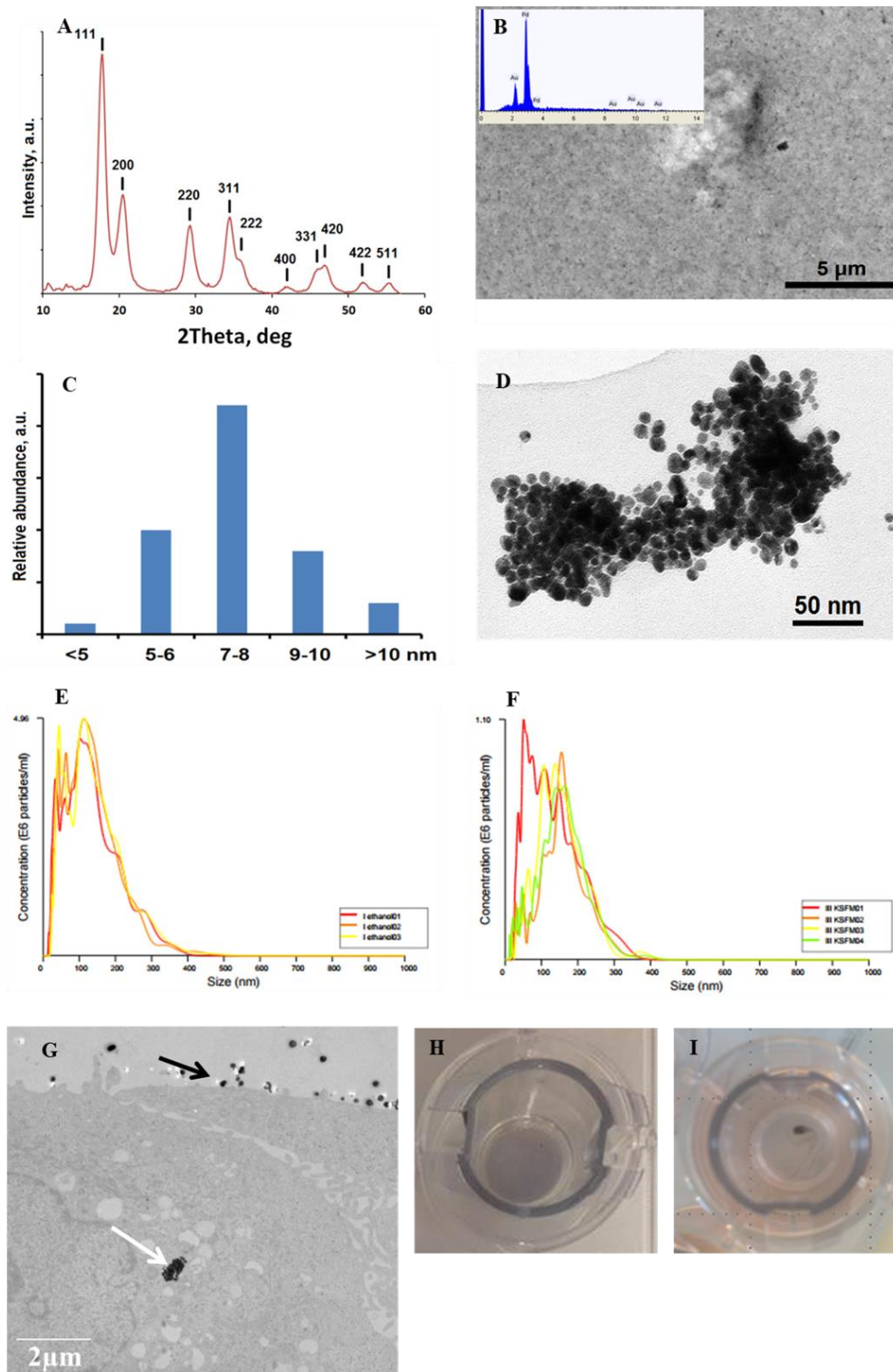


Figure 13. Characterization of Pd-NP (A-F), Pd-NP uptake (G), mucociliary clearance effects (H&I) after Pd-NP exposures using the XposeALI system.

A: X-ray powder diffraction pattern of the produced Pd-NP. B: Scanning electron microscope view of the Pd-NP collected on a gold-sputtered carbon filter with an EDS spectrum as in set; Bar scale: 5 μm . C: Distribution of Pd-NP size; 6–10 nm with a distinctly pronounced maximum at 8 nm. D: Transmission electron microscope views of the produced Pd nanoparticles; Bar scale: 50 nm. E: Hydrodynamic size of the aggregates in ethanol; determined by Laser reflection microscopy; 138 ± 3 nm. F: Hydrodynamic size of the aggregates in KSFM medium; determined by Laser reflection microscopy; 151 ± 6 nm. G: The internalization of Pd-NP in 3 mins exposed 3D model and incubated for 24 hours post exposure; most of Pd-NP localized on the cell surface (black arrow), while few Pd-NP localized inside the cells (white arrow); Bar scale: 2 μm . H: The models were exposed to high dose (650 ng/cm²) of Pd-NP and even distribution was observed. I: The Pd-NP redistributed and accumulated at one spot within 24 hours, indicating existing mucociliary clearance.

The particles used in this study were proved to be high purity Pd metal by X-ray diffraction and EDS analysis (Figure 13A&B). The Pd-NP was agglomerated when drying, but easily formed a uniform aerosol with a narrow size distribution between 6–10 nm (Figure 13C). The hydrodynamic size of Pd-NP in ethanol and KSFM medium were 138 ± 3 and 151 ± 6 nm, respectively (Figure 13E&F).

The Pd-NP exposure dose in the model was analyzed by ICP-MS and the three doses of Pd-NP used were 250 ng/cm^2 (low), 400 ng/cm^2 (medium) and 650 ng/cm^2 (high). After Pd-NP exposure with XposeALI system, we found most of the Pd-NP immobilized on the cell surface with a few Pd-NP inside the cells, and there were no differences between different incubation time post exposure (Figure 13G). Interestingly, after exposure, Pd-NP were spread evenly on the cell surfaces (Figure 13H), but after 24 hours of incubation, the Pd-NP were redistributed and accumulated at one spot (Figure 13I). This phenomenon may indicate an existing mucociliary clearance effect of the model.

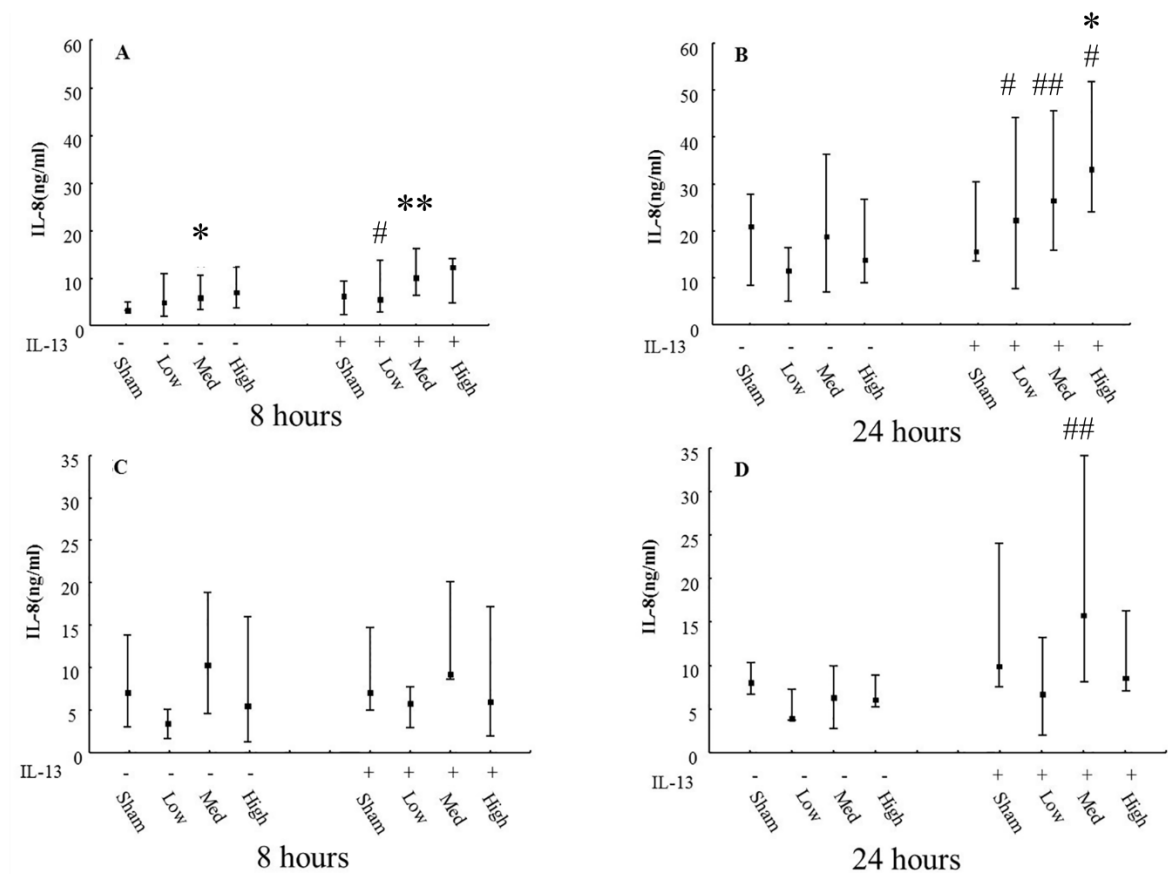


Figure 14. Release of IL-8/CXCL-8 after Pd-NP exposures using the XposeALI system.

Levels of IL-8/CXCL-8 in basal medium in normal or 1 ng/ml IL-13 treated models (N = 9) after exposure to different exposure doses of Pd-NP and incubated for 8 hours (A) and 24 hours (B). Levels of IL-8/CXCL-8 in apical medium in normal or 1 ng/ml IL-13 treated models (N = 9) after exposure to different exposure doses of Pd-NP and incubated for 8 hours (C) and 24 hours (D). Exposure: Sham: normal air and clean system; Low: 250 ng/cm²; Med: 400 ng/cm²; High: 650 ng/cm². Data presented as median and 25th-75th percentiles; *: P<0.05 VS Sham exposure; #, ##: P<0.05, 0.01 VS normal model.

Before and after exposure, a viability of models above 95 % is accepted. The apoptotic rate was 1 -30 % with no difference between sham and Pd-NP exposure neither between different normal nor different chronic bronchitis-like 3D models.

After 24 hours incubation post Pd-NP exposure, the level of IL-8/CXCL-8 in BM from chronic bronchitis-like 3D model was higher than normal model (Figure 14B). The same pattern was shown in BM from unexposed model. There was a tendency of Pd-NP dose-dependently increase of BM IL-8/CXCL-8 secretion after 8 hours incubation in both normal and chronic bronchitis-like 3D models (Figure 14A). However, after 24 hours incubation, this tendency only persisted in chronic bronchitis-like 3D model (Figure 14B). The BM level of IL-8/CXCL-8 was significantly higher after 24 hours incubation than after 8 hours (Figure 14A&B), while there was no difference between these two time points regarding AM IL-8/CXCL-8 levels (Figure 14C&D). Worth mentioning in all models, after Pd-NP exposure, the release of MMP-9 in BM were significantly lower than in AM (data not shown).

4.3 PAPER III

In Paper III, the aim was to investigate the inflammatory and oxidative stress responses to exposure to aerosolized carbon nanoparticles (CNP) in normal and chronic bronchitis-like models (IL-13 induced) using physiologically relevant primary bronchial epithelial cells (PBEC) developed air liquid interface (ALI) *in vitro* model.

The PBEC were cultured at ALI and considered as normal model (PBEC-ALI), and 1 ng/ml IL-13 treatment was used to develop chronic bronchitis-like model (PBEC-ALI/CB). XposeALI exposure system was used to perform 3 different doses of CNP exposure. Exposure to normal air and clean system served as a control. After 6 hours and 24 hours poster exposure, the protein levels of CXCL-8 and MMP-9 in BM were detected by ELISA. Transcript expression of pro-inflammatory, oxidative stress and tissue injury/repair markers were assessed by RT-PCR.

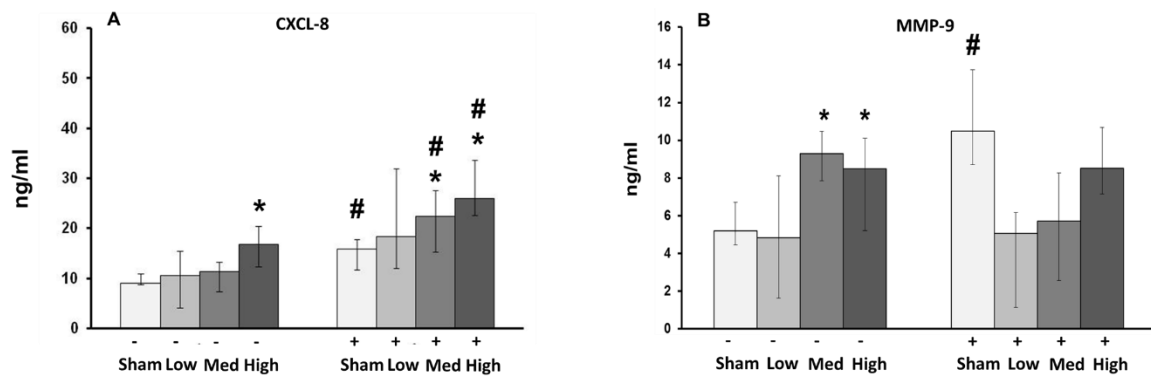


Figure 15. Carbon nanoparticles (CNP) induced protein secretion in basal media (BM) of normal (PBEC-ALI) and interleukin-13 (IL-13) induced chronic bronchitis-like mucosa (PBEC-ALI/CB).

Concentration of CXCL-8 (A) and MMP-9 (B) was measured using ELISA from BM, collected from both PBEC-ALI (-) and PBEC-ALI/CB (+) after incubation of 24 hours following exposure to sham (clean air), low (2.5 $\mu\text{g}/\text{cm}^2$), medium (9.4 $\mu\text{g}/\text{cm}^2$) and high (14 $\mu\text{g}/\text{cm}^2$) doses of CNP. Data presented as median and 25th -75th percentiles (n=9). *: $p < 0.05$ vs sham, #: $p < 0.05$ vs PBEC-ALI at corresponding dose.

In PBEC-ALI, CXCL-8 secretion was significantly increased at 24 hours post exposure to CNP (high dose) compared to sham exposure (Figure 15A, left panel). In PBEC-ALI/CB, 24 hours exposure to the two highest doses of CNP resulted in increased CXCL-8 secretion compared to sham exposure (Figure 15A, right panel). As for the secretion of MMP-9, in PBEC-ALI, MMP-9 levels in BM were increased significantly at 24 hours following exposure to both highest doses of CNP (Figure 15B, left panel). Additionally, CXCL-8 secretion was significantly increased after exposure to sham and the two highest doses of CNP in PBEC-ALI/CB model compared to corresponding PBEC-ALI model (Figure 15A). In PBEC-ALI/CB exposure to sham induced a higher release of MMP-9 levels compared to sham exposed PBEC-ALI 24 hours post exposure (Figure 15B, left panel). At 6 hours following CNP exposure, both CXCL-8 and MMP-9 levels remained unchanged at all CNP doses in both PBEC-ALI and PBEC-ALI/CB (data not shown).

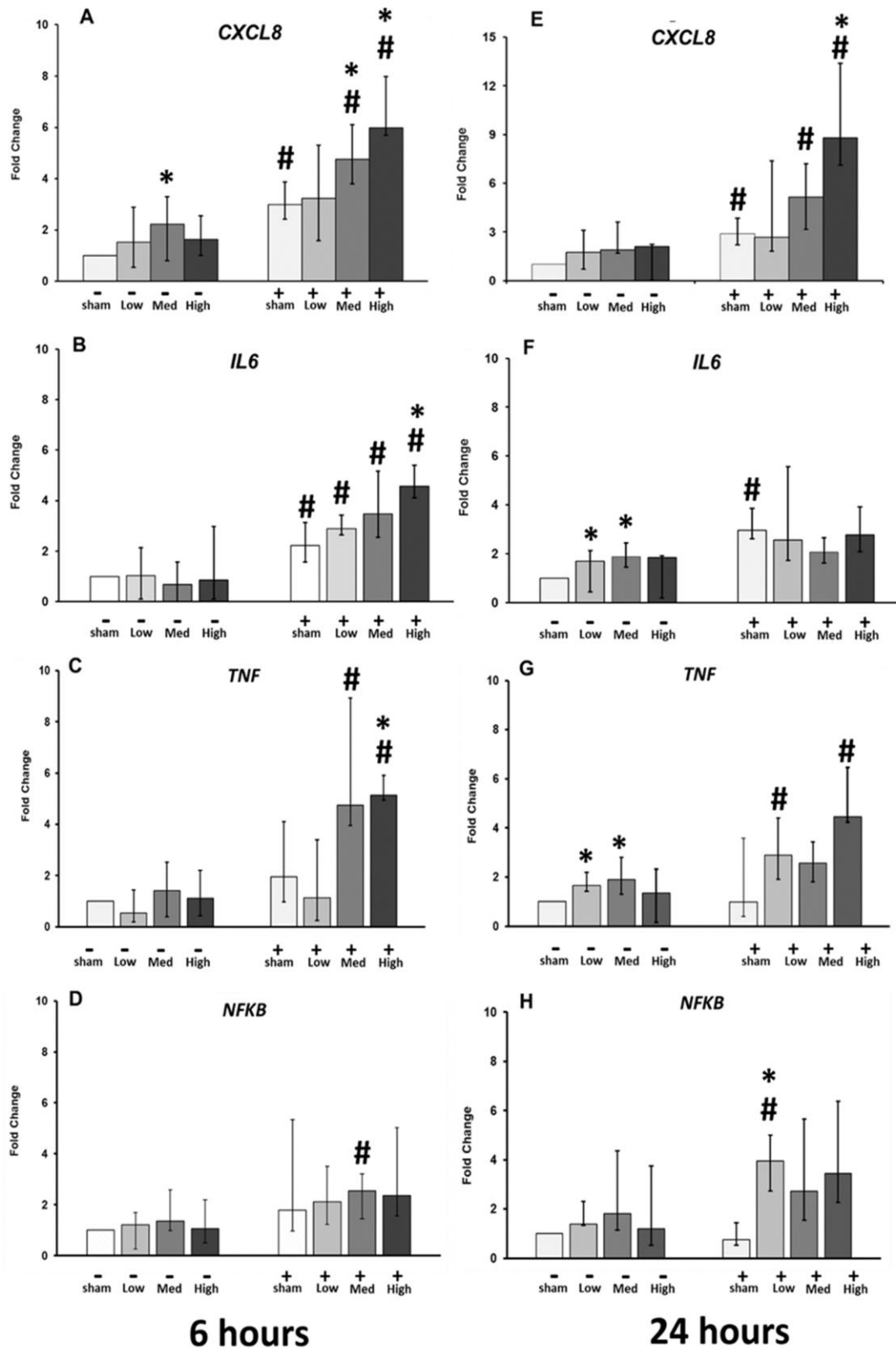


Figure 16. Transcript expression of pro-inflammatory markers in normal (PBEC-ALI (-)) and interleukin-13 (IL-13) induced chronic bronchitis-like mucosa models (PBEC-ALI/CB (+)) after 6 hours (A-D) and 24 hours (E-H) incubation following exposure to carbon nanoparticles (CNP).

Fold change of *CXCL8* (A and E), *IL6* (B and F), *TNF α* (C and G) and *NFKB* (D and H) 6 hours and 24 hours post exposure to sham (clean air), low (2.5 $\mu\text{g}/\text{cm}^2$), medium (9.4 $\mu\text{g}/\text{cm}^2$) and high (14 $\mu\text{g}/\text{cm}^2$) CNP doses. Data presented as median and 25th -75th percentiles (n=6). *: p<0.05 vs sham, #: p<0.05 vs PBEC-ALI at corresponding dose.

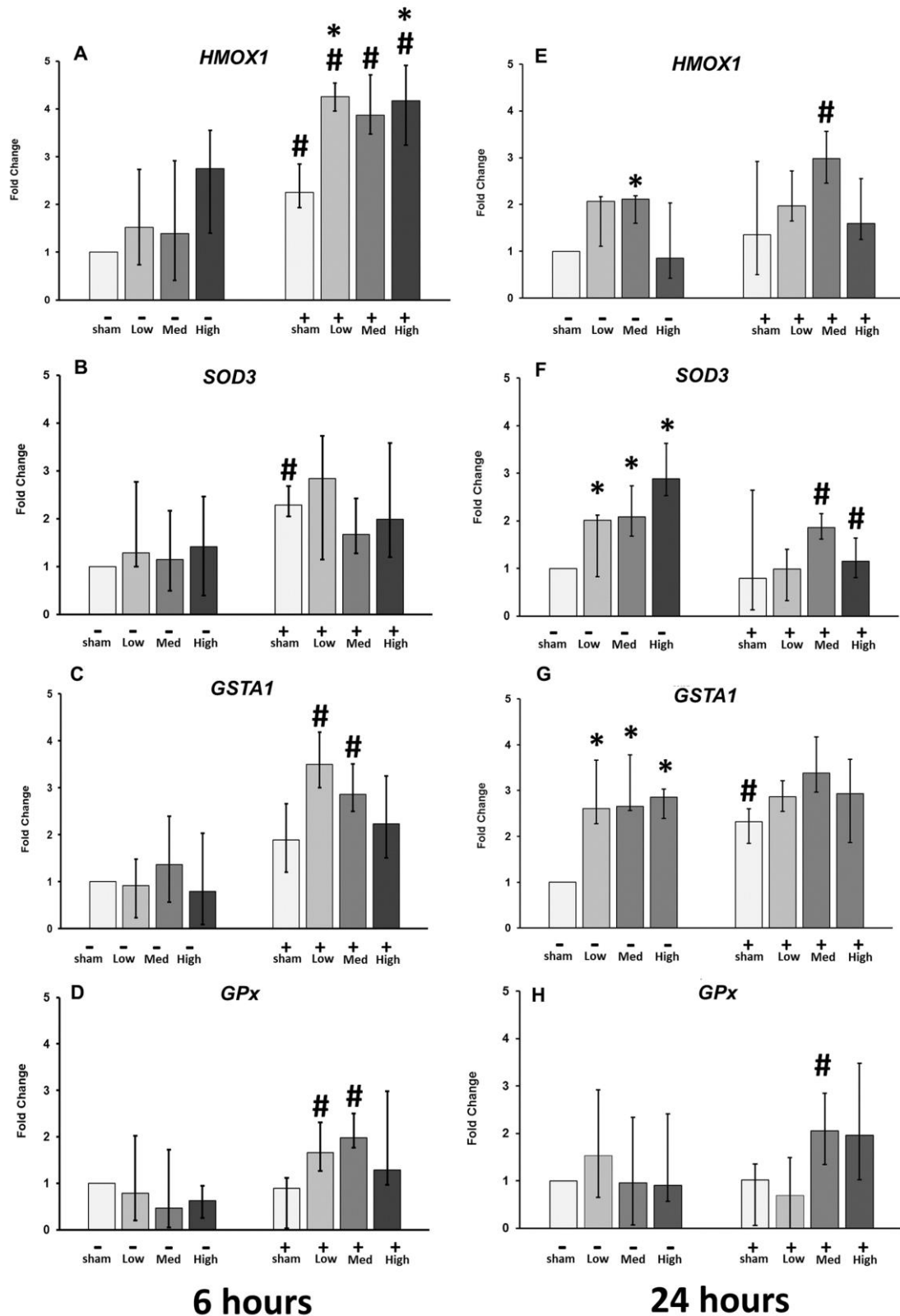


Figure 17. Transcript expression of oxidative stress markers in normal (PBEC-ALI (-)) and interleukin-13 (IL-13) induced chronic bronchitis-like mucosa models (PBEC-ALI/CB (+)) after 6 hours (A-D) and 24 hours (E-H) incubation following exposure to carbon nanoparticles (CNP).

Fold change of *HMOX1* (A and E), *SOD3* (B and F), *GSTA1* (C and G) and *GPx* (D and H) 6 hours and 24 hours post exposure to sham (clean air), low (2.5 $\mu\text{g}/\text{cm}^2$), medium (9.4 $\mu\text{g}/\text{cm}^2$) and high (14 $\mu\text{g}/\text{cm}^2$) CNP doses. Data presented as median and 25th -75th percentiles (n=6). *: p<0.05 vs sham, #: p<0.05 vs PBEC-ALI at corresponding dose.

Transcript expression of pro-inflammatory markers (*CXCL8*, *IL6*, *TNF α* , *NFkB*; Figure 16) and oxidative stress genes (*HMOX1*, *SOD3*, *GSTA1*, *GPx*; Figure 17) were assayed from both PBEC-ALI and PBEC-ALI/CB models at 6 hours and 24 hours post exposure to sham and aerosolized CNP at 3 different doses. Exposure to aerosolized CNP resulted in increased transcript expression of *CXCL8*, *IL6* and *TNF α* after 6 hours in PBEC-ALI/CB (highest dose vs sham; Figures 16A-C, right panel). Additionally, 24 hours following exposure to CNP in the PBEC-ALI, the mRNA expressions of *IL6* and *TNF α* were significantly increased (low and medium doses vs sham; Figure 16F&G, left panel), while expressions of *CXCL8* and *NFkB* remained unaltered after exposure to any CNP doses (Figure 16E&H, left panel). Only *HMOX1* transcript expression was increased 6 hours post CNP exposure in the PBEC-ALI/CB (low and high doses vs sham; Figure 17A, right panel). After 24 hours culturing, expression of *HMOX1* (low dose vs sham; Figure 17E, left panel) as well as *SOD3* and *GSTA1* (all three doses vs sham; Figure 17F&G, left panel) were induced by CNP exposure in PBEC-ALI.

After both 6 hours and 24 hours following CNP exposure, *CXCL8* expression increased more in PBEC-ALI/CB compared to PBEC-ALI (sham, medium and high doses, Figure 16A&E). Also, *IL6* mRNA expression was significantly higher in PBEC-ALI/CB compared to PBEC-ALI 6 hours after exposure to both sham and CNP at all 3 different doses (Figure 16B). Besides, CNP exposure induced a higher expression of *TNF α* in PBEC-ALI/CB than in PBEC-ALI (6 hours, medium and high dose; 24 hours, low and high dose; Figure 16C&G). The mRNA expression of *HMOX1* was significantly higher in PBEC-ALI/CB compared to PBEC-ALI after exposure to both sham and CNP at all 3 different doses (Figure 17A). As for *SOD3*, an enhanced mRNA expression in PBEC-ALI/CB compared to PBEC-ALI could only be found 6 hours post sham exposure (Figure 17B), while a decreased expression 24 hours post CNP exposure (medium and high doses; Figure 17F) were observed in PBEC-ALI/CB compared with PBEC-ALI. Both *GSTA1* and *GPx* were significantly increased 6 hours after CNP exposure at the two lowest doses in PBEC-ALI/CB compared with PBEC-ALI (Figure 17C&D).

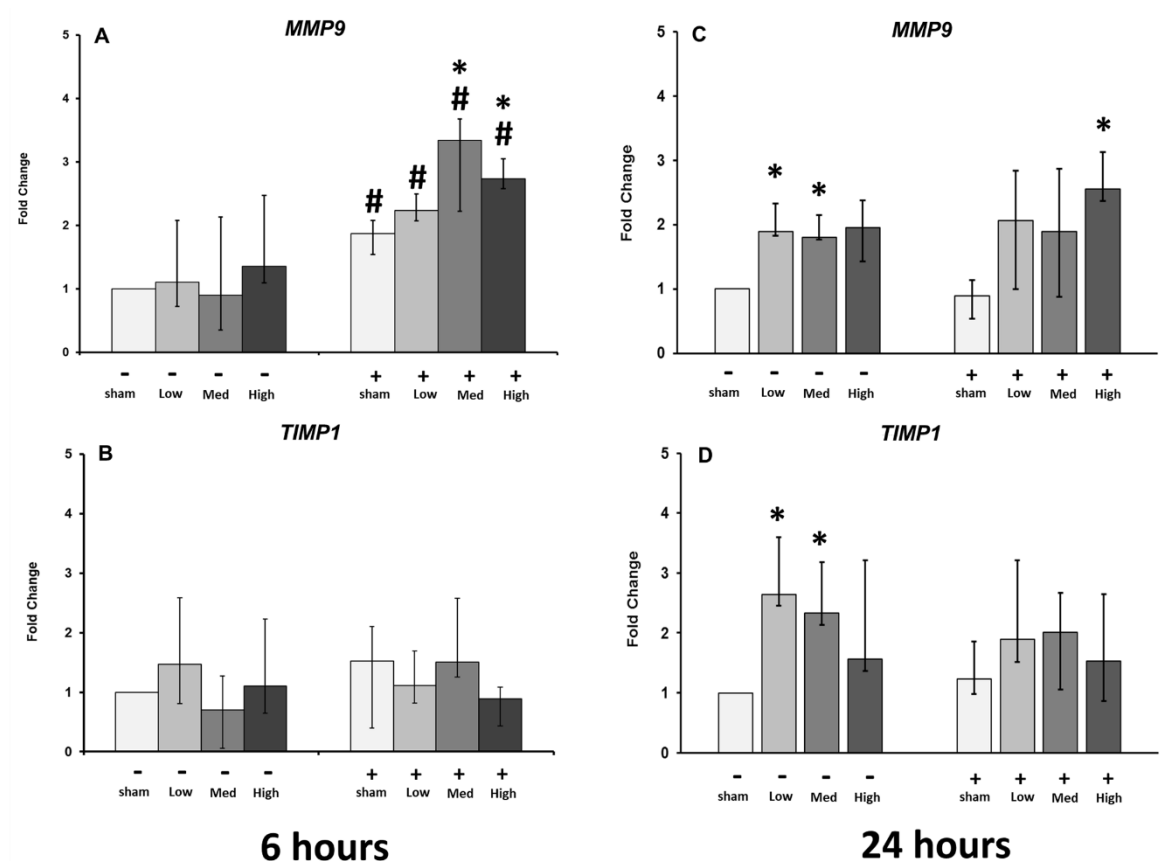


Figure 18. Transcript expression of tissue injury/repair markers in normal (PBEC-ALI (-)) and Interleukin-13 (IL-13) induced chronic bronchitis-like mucosa (PBEC-ALI/CB (+)) after 6 hours (A&B) and 24 hours (C&D) incubation following exposure to Carbon nanoparticles (CNP).

Fold change of *MMP9* (A and C), and *TIMP1* (B and D) 6 hours and 24 hours post exposure to sham (clean air), low (2.5 $\mu\text{g}/\text{cm}^2$), medium (9.4 $\mu\text{g}/\text{cm}^2$) and high (14 $\mu\text{g}/\text{cm}^2$) CNP doses. Data presented as median and 25th -75th percentiles (n=6). *: $p < 0.05$ vs sham, #: $p < 0.05$ vs PBEC-ALI at corresponding dose.

Six hours after exposure to the two highest doses of CNP the expression of *MMP9* were significantly upregulated in PBEC-ALI/CB while corresponding *TIMP1* expression did not change (Figure 18A&B, right panel). After 24 hours culturing the expression of both *MMP9* and *TIMP1* increased significantly after exposure to the two lowest doses of CNP in PBEC-ALI (Figure 18C&D, right panel). After 6 hours post-exposure to both sham and all three concentrations CNP exposure, resulted in a significantly higher *MMP9* expression in PBEC-ALI/CB compare to PBEC-ALI (Figure 18A).

4.4 PAPER IV

In Paper IV, the aim was to build multi-cellular model consisting of human primary bronchial epithelial cells (PBEC) cultured at ALI with and without the addition of macrophages (MQ, THP-1 cell differentiated macrophage), followed by diesel exhaust particles (DEP) exposure to identify crosstalk between airway epithelial cells and MQ.

The PBEC cultured at ALI were co-cultured without (PBEC-ALI) and with MQ (PBEC-ALI/MQ). The models were exposed to aerosolized DEP using Xpose^{ALI}. Exposure to normal air and clean system served as a control. The CXCL8 and IL-6 were measured in BM by ELISA. The mRNA expression of inflammatory, oxidative stress and MQ polarization markers were measured by RT-PCR. The surface/mRNA expression of TLR2/TLR4 was detected by FACS and RT-PCR, respectively.

For further detail see paper IV. All the figures have been published and permitted to be used here[176].

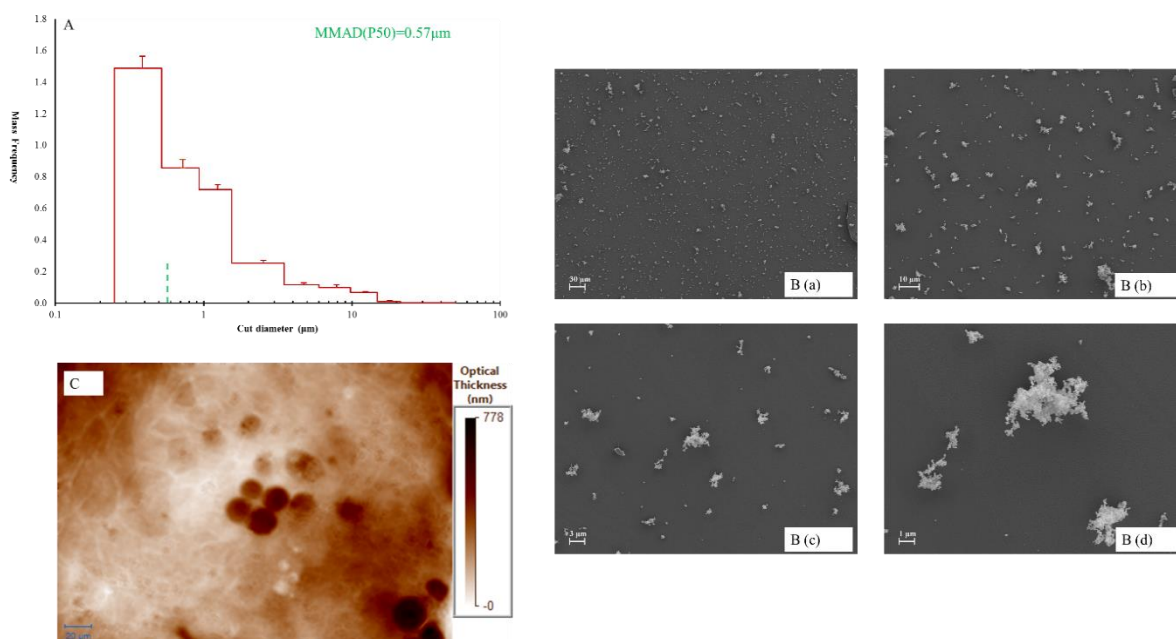


Figure 19. Characterization and uptake of diesel exhaust particulates (DEP) .

A: Mass frequency of DEP in different size ranges measured by the 9-stage Marple Cascade Impactor; MMAD (P50): Mass Median Aerodynamic Diameter; The MMAD (P50) of DEP was estimated to be 0.57µm. B: SEM image of DEP soot with different magnifications; B(a): 500X, bar: 30 µm; B(b): 2000X, bar: 10 µm; B(c): 5000X, bar: 3 µm; B(d): 15000X, bar: 1 µm. C: Quantitative phase image of the apical side of PBEC-ALI/MQ, which reflects the distribution of mass across the field; Bar: 20µm.

According to a 9-stage Marple Cascade Impactor analysis, the mass median aerodynamic diameter (MMAD, P50) of DEP aerosols used in this study was 0.57µm (Figure 19A). After XposeALI exposure, the SEM figures indicated an evenly distribution of DEP with similar agglomerated structure as previous reported[177] (Figure 19B). The DEP is free form LPS based on results from LAL testing (data not shown). By plugging Quadri-Wave Lateral Shearing Interferometry (QWLSI) onto microscope, we found, clearly distinguished from epithelial cells, MQ that appeared contrasted with some dense parts which may indicate the engulfment of DEP particles (Figure 19C). The dose of DEP after exposure was detected by rinsing the exposed insert with 99% ethanol and detecting with spectrophotometric technique. The DEP dose in this study was 3.4 (low), 5 (medium) and 12.7 (high) µg/cm² respectively.

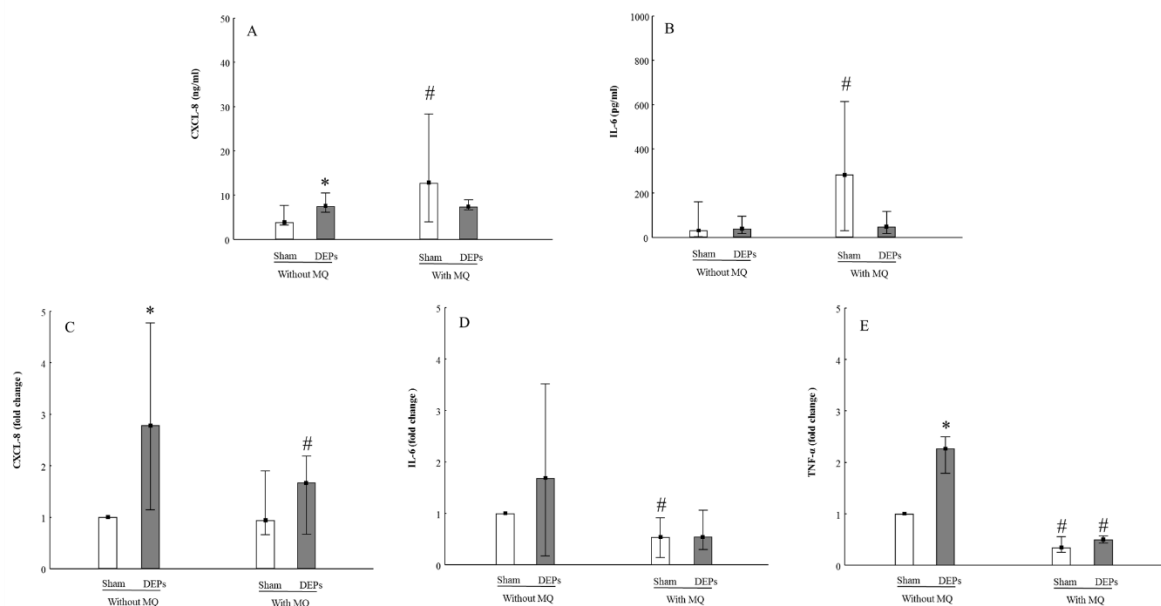


Figure 20. Release and mRNA expression of inflammatory biomarkers in models after exposures to diesel exhaust particulates (DEP) using the XposeALI system.

Levels of CXCL-8 (A) and IL-6 (B) secretion in basal medium in PBEC-ALI and PBEC-AL/MQ (N=9) after exposure to DEP and incubated for 24 hours; Fold change of *CXCL8* (C), *IL6* (D) and *TNFα* (E) mRNA expression in PBEC-ALI and PBEC-AL/MQ (N=6) after exposure to DEP and incubated for 24 hours; Exposure: sham: clean air; DEP: 12.7 $\mu\text{g}/\text{cm}^2$; Data presented as median and 25th -75th percentiles, fold change = $2^{-\Delta\Delta\text{Ct}}$ of models / $2^{-\Delta\text{Ct}}$ of sham exposed PBEC-ALI; *: $P < 0.05$ VS Sham exposure; #: $P < 0.05$ VS PBEC-ALI.

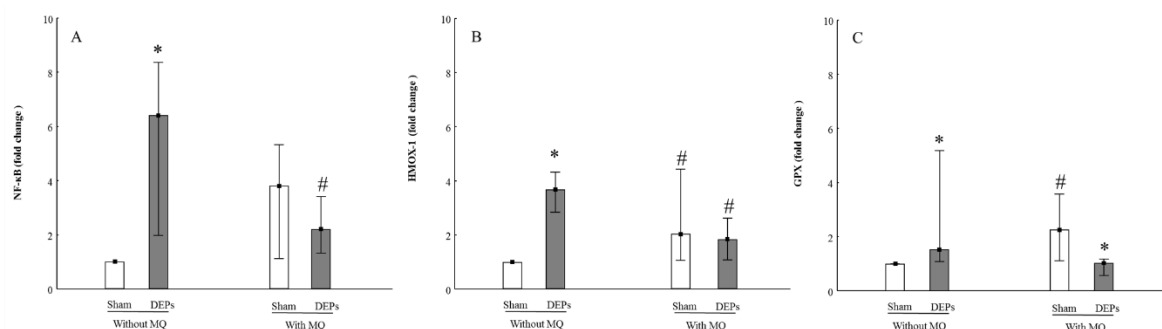


Figure 21. mRNA expression of oxidative stress related markers in models after exposures to diesel exhaust particulates (DEP) using the XposeALI system.

Fold change of *NFκB* (A), *HMOX1* (B) and *GPx* (C) mRNA expression in PBEC-ALI and PBEC-AL/MQ (N=6) after exposure to DEP and incubated for 24 hours; Exposure: sham: clean air; DEP: 12.7 $\mu\text{g}/\text{cm}^2$; Data presented as median and 25th -75th percentiles, fold change = $2^{-\Delta\Delta\text{Ct}}$ of models / $2^{-\Delta\text{Ct}}$ of sham exposed PBEC-ALI; *: $P < 0.05$ VS Sham exposure; #: $P < 0.05$ VS PBEC-ALI.

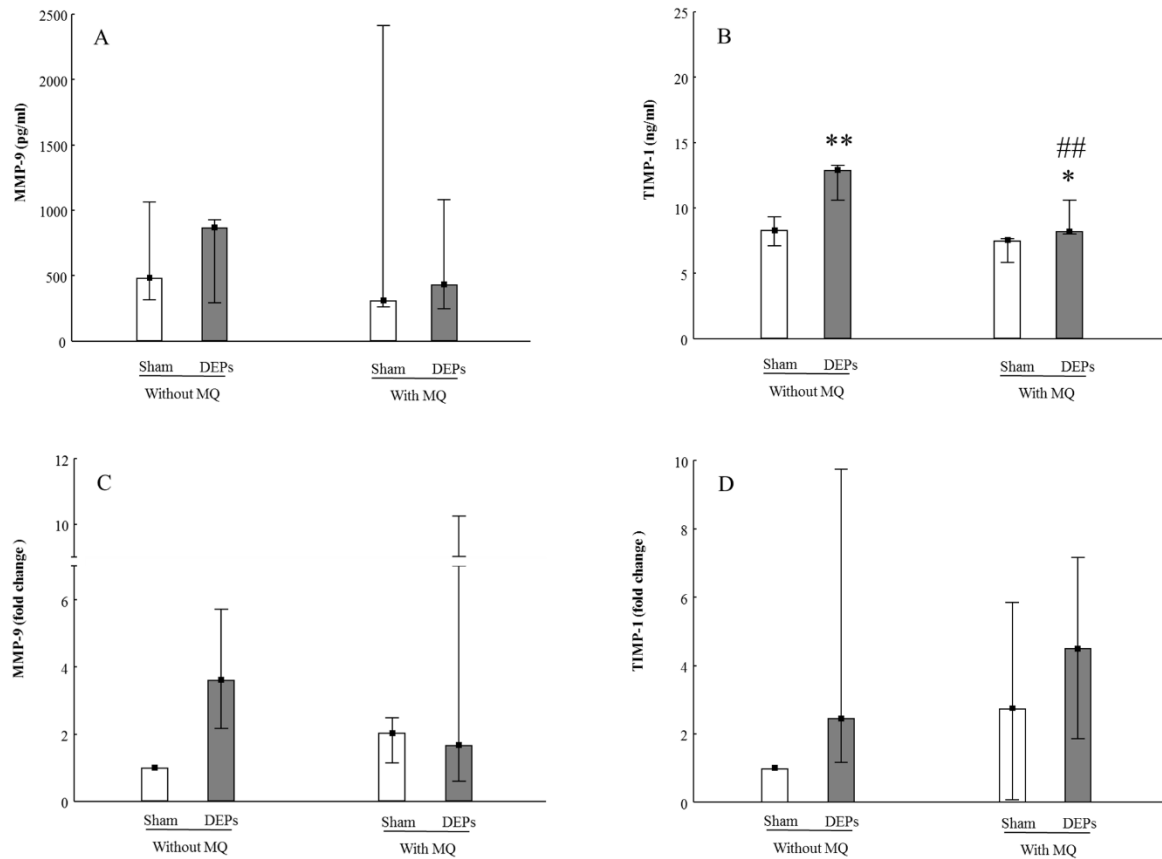


Figure 22. Release and mRNA expression of extra cellular markers in models after exposures to diesel exhaust particulates (DEP) using the XposeALI system.

Levels of MMP-9 (4A) and TIMP-1 (4B) secretion in basal medium in PBEC-ALI and PBEC-AL/MQ (N=9) after exposure to DEP and incubated for 24 hours; Fold change of *MMP-9* (4C) and *TIMP-1* (4D) mRNA expression PBEC-ALI and PBEC-AL/MQ (N=6) after exposure to DEP and incubated for 24 hours; Exposure: sham: clean air; DEP: 12.7 $\mu\text{g}/\text{cm}^2$; Data presented as median and 25th-75th percentiles, fold change = $2^{-\Delta\Delta\text{Ct}}$ of models / $2^{-\Delta\Delta\text{Ct}}$ of sham exposed PBEC-ALI; *, **: P<0.05, 0.01 VS Sham exposure; ##: P<0.01 VS PBEC-ALI.

The secretion of CXCL-8 (Figure 20A) and IL-6 (Figure 20B) and the mRNA expression of *HMOX1* (Figure 21B) and *GPx* (Figure 21C) were upregulated, while *IL6* (Figure 20D) and *TNF α* (Figure 20E) transcript were downregulated in PBEC-ALI/MQ compared to PBEC-ALI as a consequence of sham exposure. In PBEC-ALI, CXCL-8 (Figure 20A) and TIMP-1 (Figure 22B) secretion as well as *CXCL8* (Figure 20C), *TNF α* (Figure 20E), *NF κ B* (Figure 21A), *HMOX1* (Figure 21B) and *GPx* (Figure 21C) mRNA expression were significantly increased after DEP exposure compared to sham exposure. The secretion of TIMP-1 (Figure 22B) and the expression of *CXCL8* (Figure 20C), *TNF α* (Figure 20E), *NF κ B* (Figure 21A) and *HMOX1* (Figure 21B) were decreased in PBEC-ALI/MQ compared to PBEC-ALI indicating that MQ attenuate the effects of DEP exposure. The levels of TNF- α secretion were under detection limit (data not shown).

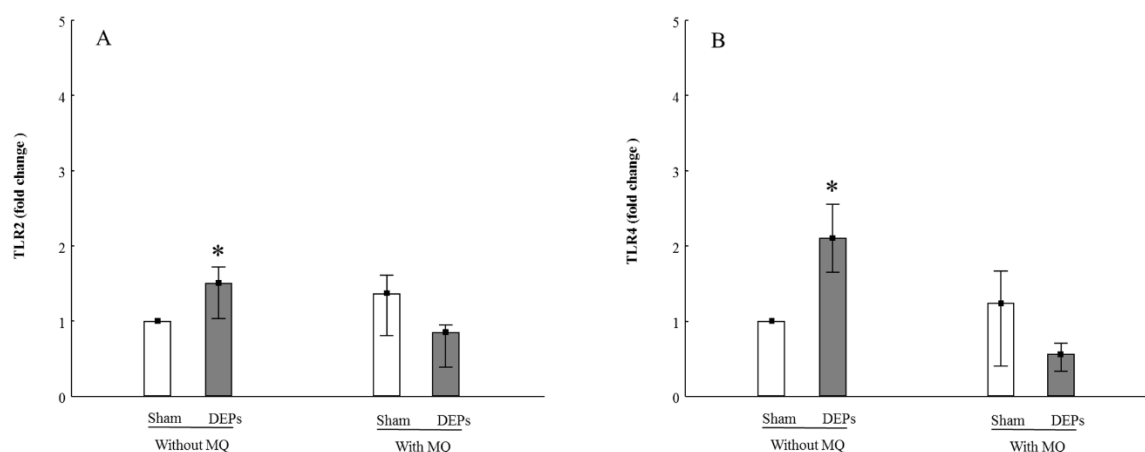


Figure 23. mRNA expression of Toll-like receptor in models after diesel exhaust exposures to particulates (DEP) using the XposeALI system.

Fold change of *TLR2* (A) and *TLR4* (B) mRNA expression in PBEC-ALI and PBEC-ALI/MQ (N=6) after exposure to DEP and incubated for 24 hours; Exposure: sham: clean air; DEP: 12.7 $\mu\text{g}/\text{cm}^2$; Data presented as median and 25th-75th percentiles, fold change = $2^{-\Delta\text{Ct}}$ of models / $2^{-\Delta\text{Ct}}$ of sham exposed PBEC-ALI; *: $P < 0.05$ VS Sham exposure.

The mRNA expressions of *TLR2* and *TLR4* were increased by DEP exposure in PBEC-ALI (Figure 23).

The surface expression of TLR was detected by FACS. As mentioned in section 3.2.8.2, anti-CD68 antibody was used to distinguish between PBEC (CD68⁻) and MQ (CD68⁺) in PBEC-ALI/MQ model. The ratio of PBEC and MQ was 10:1(data not shown) which was the same ratio as we seeded. Then TLR2 and TLR4 surface expressions on PBEC and MQ were detected separately with FACS analysis (Figure 24). Sham exposure attenuated TLR2 and TLR4 surface expression on PBEC in PBEC-ALI/MQ than in PBEC-ALI (Figure 24A(c)&(d)) while there was no such alternation after DEP exposure. In PBEC-ALI, the PBEC surface expression of TLR4 decreased after DEP exposure compared to sham exposure (Figure 24A(d)). When co-cultured with MQ, the PBEC surface expression of TLR4 decreased while TLR2 increased after DEP exposure compared with sham exposure (Figure 24A(c)&(d)). As for MQ in PBEC-ALI/MQ, DEP exposure led to the same surface expression pattern of TLR2 and TLR4 as PBEC (Figure 24B(c)&(d)).

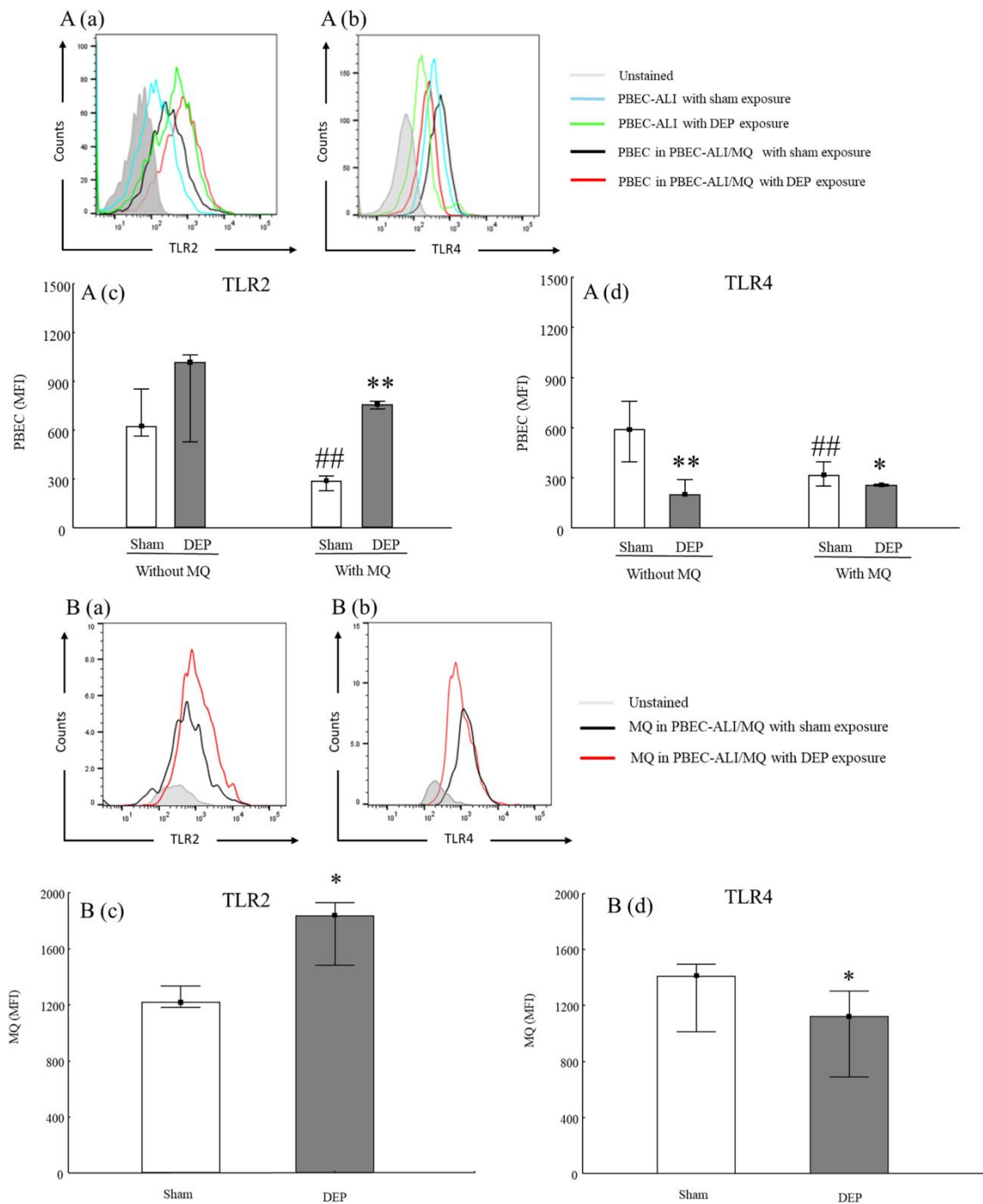


Figure 24. Expression of TLR2 and TLR4 on the surface of primary bronchial epithelial cells (PBEC) and THP-1 cell derived macrophage (MQ) in models after diesel exhaust exposures to particulates (DEP) using the XposeALI system.

PBEC (A) and MQ (B) were identified by anti-CD 68-PE-Cy7. A representative of 9 experiments is shown (A (a&b), B (a&b)); The expression of TLR2 (A (c)) and TLR4 (A (d)) on surface of PBEC in PBEC-ALI and PBEC-ALI/MQ was presented as median and 25th - 75th percentiles (N=9); *, **: P<0.05, 0.01 VS Sham exposure; ##: P<0.01 VS PBEC-ALI. The expression of TLR2 (B (c)) and TLR4 (B (d)) on surface of MQ which have been co-cultured with PBEC was presented as median and 25th -75th percentiles (N=9); *: P<0.05 VS Sham exposure; Exposure: sham: clean air; DEP: 12.7 µg/ cm².

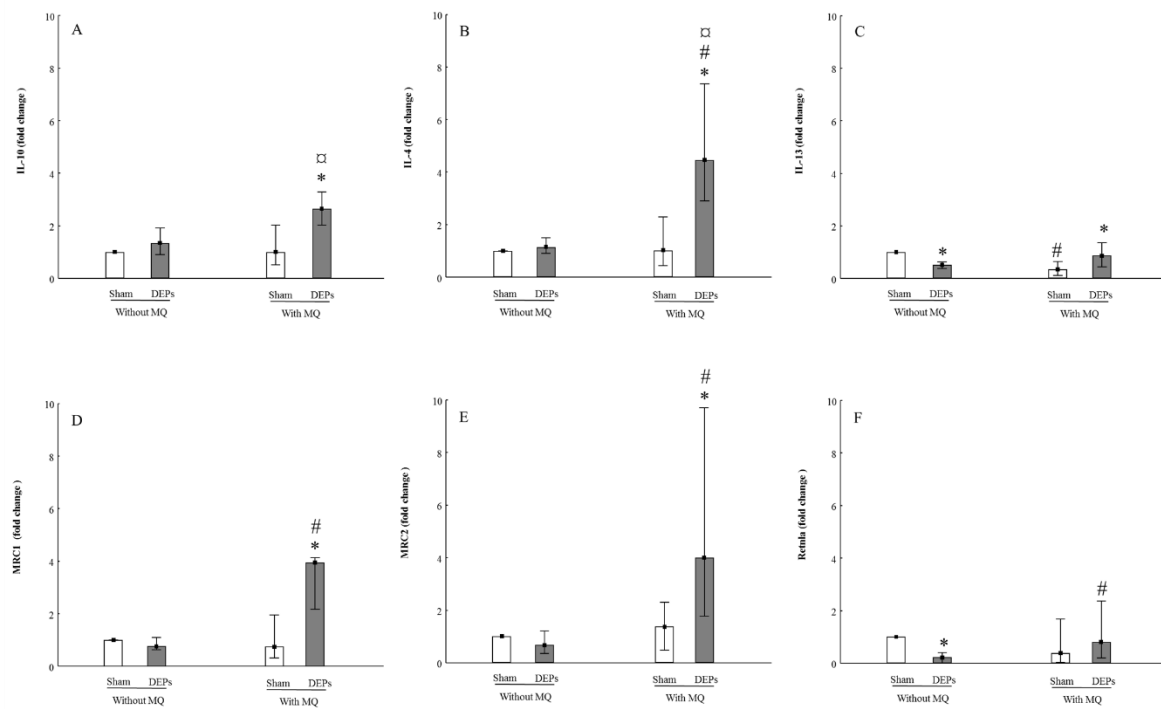


Figure 25. mRNA expression of M2 macrophages markers after diesel exhaust exposures to particulates (DEP) using the XposeALI system.

Fold change of *IL10* (7A), *IL4* (7B), *IL13* (7C), *MRC1* (7D), *MRC2* (7E) and *RETNLA* (7F) mRNA expression in PBEC-ALI and PBEC-ALI/MQ (N=6) after exposure to DEP and incubated for 24 hours; Exposure: sham: clean air; DEP: 12.7 $\mu\text{g}/\text{cm}^2$; Data presented as median and 25th-75th percentiles, fold change = $2^{-\Delta\text{Ct}}$ of models / $2^{-\Delta\text{Ct}}$ of sham exposed PBEC-ALI; *: P<0.05 VS Sham exposure; #: P<0.05 VS PBEC-ALI; □: P<0.05 VS Sham exposed PBEC-ALI.

The *IL4* (Figure 25B), *MCR1* (Figure 25D), *MRC2* (Figure 25E), and *RETNLA* (Figure 25F) mRNA expression increased more in PBEC-ALI/MQ compared to PBEC-ALI following DEP exposure. The DEP exposure increased all M2 macrophage transcription markers except *RETNLA* compare with sham exposure in PBEC-ALI/MQ (Figure 25). Exposed MQ monoculture only with after DEP exposure did not alternate mRNA expression of M2 macrophages markers (data not shown).

5 GENERAL DISCUSSION

The lungs are continuously exposed to a wide variety of airborne particles, antigens and toxins that affects human immune system leading to innate and adaptive immune responses as well as pro-inflammatory, anti-inflammatory and oxidative stress reactions. On one hand, lung serves as the primary port of entry for inhaled agents implying risk of development of pulmonary diseases. On the other hand, the lungs also are easily accessible for targeted drug, like bronchodilators and glucocorticosteroids.

The main objective of this thesis was to develop physiological relevant multi-cellular models of normal and chronic bronchitis-like airway mucosa in order to study cell-cell interactions, inflammatory and oxidative stress mechanisms induced by exposures to different particles[169]. We used immune competent cells like macrophages and structural cells like epithelial cells both *ex vivo* and *in vitro* and exposed them to different organic material and ultrafine or nanoparticles. A second focus was to explore innate immune responses by studying Toll-like receptors and how they can precisely modulate cellular inflammatory responses.

5.1 CELL CULTURE MODELS

The Swedish government has commissioned several agencies to draw up strategies for replacement, reduction and refinement (3R) of animal experiments according to the regulatory letter of 2016. The 3R is also taken into account during evaluation of substances within the European chemical regulation REACH, and 3R must comply with the requirements in DIRECTIVE 2010/63/EU on the protection of animals used for scientific purposes. Because of these novel instructions development and evaluation of advanced and realistic *ex vivo* and *in vivo*-like lung models at different levels are urgently needed.

Alveolar macrophage (AMQ) are the major immune-competent cell population in human lung [29] and their main function are to recognize potential pathogens or toxins entering into the lung[178]. They orchestrate airway inflammation by production of different kinds of cytokines and chemokines. Studies from our group have shown that the number of macrophages in bronchoalveolar lavage (BAL) are elevated in smokers without and with COPD compared with non-smokers[179], and that the number of macrophages was associated with the degree of severity of COPD[180]. Therefore, in Paper I, we isolated AMQ from BAL fluid from non-smokers and smokers with COPD and stimulated the AMQ *ex vivo* to elucidate how Toll-like receptors (TLR) expression is altered following exposure to different stimuli.

Due to the new directives physiologically relevant *in vivo*-like model systems are becoming increasingly interesting for studying molecular mechanisms of pulmonary diseases. As lung is a primary contact organ, bronchial epithelial cells under air-liquid interface (ALI) conditions resemble the airway wall as far as baso-apical orientation, ciliation and mucus production is concerned. In Paper II and Paper III we established airway mucosal 3D model by human primary bronchial epithelial cells (PBEC) and cultured them at ALI with and without fibroblasts, which allows the model to differentiate into ciliated cells, mucus producing cells, club cells and basal cells. By the addition of IL-13, a chronic bronchitis-like model containing increased numbers of mucus producing cells was built. Both models can mimic the bronchial epithelium in health and disease conditions *in vivo* to study the toxicity of inhaled substances.

Mono-culture of epithelial cells or macrophages responded differently to xenobiotics when compared with co-cultures these cell types[181]. In both upper and lower airways, macrophage abundantly reside along the epithelium, and facilitate interaction with bronchial epithelial cells[182]. The cell to cell contacts of macrophage and epithelium are very important in modulating macrophage phenotype and function[183]. In a pilot study included in Paper IV, we exposed the PBEC-ALI models to DEP and then co-culturing with MQ placed underneath the models. However, in these models where MQ and PBEC-ALI had no direct contact, but only indirectly with possibility to communicate through mediators, we did not see any effect of the presence of MQ after DEP exposure. Hence, the direct contact between the two types of cells seems very important in response to particle exposure. Therefore, in Paper IV, when culturing MQ on the apical surface of epithelial cell which allows direct contact between the two cell types, mimics the *in vivo* situation and enables crosstalk between the cells.

5.2 DIFFERENT STIMULI

Chronic exposure to inhaled agents such as cigarette smoke, ambient dust, and particulate matter (PM) from traffic emissions or air pollution is associated with pulmonary and systemic inflammation which eventually may result in pulmonary injuries. Pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) from endotoxin of Gram-negative bacteria and peptidoglycans (PGN) from Gram-positive bacteria constitute pro-inflammatory stimuli and may cause airway inflammation[52]. Nanoparticles are more potent pro-inflammatory stimuli and induce more cell damage than the same material of larger size[184]. Because their high lung deposition efficiency and great specific surface area[185, 186], they can combine with other substances to avoid the pulmonary clearance and subsequently cause respiratory toxicity. Exposure to nanoparticles like Pd-NP and CNP have been shown to induce cardiovascular, pulmonary or renal impairments in rodent models[168,

187]. Further, particles like DEP which are the products of diesel engine fuel incomplete combustion, has also been recognized as a risk factor for chronic bronchitis[188]. Both acute and chronic exposure to DEP can not only cause, but also increased the mortality of COPD/ asthma exacerbation, cardiovascular disease and respiratory infections[189-191]. Therefore, in this thesis, we exposed different cell cultures including AMQ *ex vivo* culture, normal/ chronic bronchitis-like 3D models (with and without fibroblast) and finally PBEC and MQ co-cultured models to these environmental stimuli (LPS, PGN, Pd-NP, CNP and DEP) in order to figure out the inflammatory and oxidative stress implement of these stimuli described above.

5.3 INFLAMMATORY AND OXIDATIVE STRESS RESPONSE

In macrophages (Paper I) we found that PGN and LPS significantly increased the secretion of CXCL-8 and TNF- α which were attenuated by budesonide in AMQ from healthy controls and smokers with and without COPD (one exception in smokers without COPD). This finding was in line with previous results from our group, where LPS, PGN and TNF stimulations of PBEC increased the release of CXCL-8 and IL-6, which was reduced by the addition of budesonide[52]. Budesonide is a glucocorticosteroid frequently used alone or in combination with β_2 -agonists in the treatment of asthma and COPD. Budesonide binds to the glucocorticosteroid receptor (GR) which is located in the cytosol. The GR-glucocorticosteroid complex then enters the nucleus and may take two paths, either activate anti-inflammatory gene transcription or inhibit pro-inflammatory gene transcription[19]. Hence, budesonide induced inhibition of LPS and PGN induced inflammatory responses could either be caused by switching off multiple inflammatory gene and inhibiting the translocated of transcription factors from cytoplasm to nucleus or switch on anti-inflammatory gene. Interestingly, in Paper I, both control and PGN-treated AMQ from smokers without COPD produced more CXCL-8 than healthy controls. Also Bud attenuate the TLR-ligand induced CXCL-8 secretion with the same exception in PGN-treated AMQ from smokers without COPD. These results may indicate that AMQ from smokers without COPD are more active and less glucocorticosteroid sensitive than AMQ from healthy controls.

In epithelial cells we detected inflammatory and oxidative stress responses to different particle (Pd-NP, CNP and DEP) exposures in all models used (PBEC-ALI, PBEC-ALI/CB and PBEC-ALI/MQ models) (Paper II-IV). We found increased secretion of CXCL-8, as well as increased mRNA expression of *CXCL8*, *TNF α* , and *NF κ B* 24 hours post exposure of PBEC-ALI to DEP (Paper IV). Similarly, previous studies showed that DEP exposure increased the levels of CXCL-8, IL-6 and TNF- α in human alveolar epithelial ALI models and in epithelial cell lines[192-194]. The Pd-NP and CNP aerosol exposure dose-dependently increased CXCL-8

release in basal medium after 24 hours in chronic bronchitis-like model (Paper II&III). This is in line with our previous study that showed increased secretion of CXCL-8 at the highest concentration of culture medium dispersed Pd-NP when culturing PBEC at submerge condition [167]. Also one study identified similar significant dose-dependent increase of *CXCL8* expression in A549 cells after 24 hours of exposure to CNP[195]. Interestingly, in Paper III, we showed that at 6 hours following CNP exposure, *IL6* and *TNFα* expression was upregulated in PBEC-ALI/CB model, while at 24 hours following CNP exposure such increase was only found in PBEC-ALI. We conclude that the pro-inflammatory effect following CNP exposure seem to appear at an earlier stage in the PBEC-ALI/CB than in PBEC-ALI. Although, the induction of *IL6* mRNA expression or *TNFα* secretion upon CNP exposure has been studied in animal model[168, 196], this time dependent regarding pro-inflammatory response and PBEC-ALI/CB respond earlier seems rarely been reported.

It is well known that both CNP and DEP can induce an production of ROS[197-199]. The excessive ROS production challenges the cellular homeostasis by disturbing the balance between ROS and antioxidant production. The ROS may trigger the translocation of Nrf2 and subsequently, hetero-dimerized with antioxidant response elements (ARE) in the nucleus. In combination with other transcription factors, the Nrf2/ARE complex induced the expression of antioxidant genes like *HMOX1*, *SOD3*, *GSTA1* and *GPx*[200]. Zarcone *et al* identified that exposure to DEP induced stress responses in PBEC ALI culture[201]. Consistently, in Paper III&IV, we found increased mRNA expression of antioxidant genes (*HMOX1*, *SOD3* and *GSTA1* upon CNP exposure; *HMOX1* and *GPx* upon DEP exposure) in PBEC-ALI 24 hours post CNP and DEP exposure. Interestingly, the induction of *HMOX1* transcript expression after 6 hours post exposure CNP exposure can only be found in PBEC-ALI/CB, which was similar as pro-inflammatory response (Paper III).

By adding Th2 cytokines like IL-13, the bronchial epithelial cells differentiate further and the number of mucus-producing cells and MU5AC production increased, which are characteristic features of chronic bronchitis[44, 202]. Also IL-13 expression is increased in central airway of smokers with chronic bronchitis[134]. Besides, in Paper II, we showed that IL-13 treatment (1 ng/ml) increased mucus-producing cell and MU5AC mRNA expression in our chronic bronchitis-like 3D model. Thus, we regard the models treated with IL-13 representing chronic bronchitis conditions (Paper II&III). We found that CXCL-8 secretion levels were higher in chronic bronchitis-like models than normal models 24 hours after sham and Pd-NP or CNP exposure. This could be an expression of more activated cells in the chronic bronchitis-like model. Also, it is possible that mucus-producing cells can produce

more CXCL-8 than ciliated cells, since there were a higher proportion of mucus-producing cells in chronic bronchitis-like mucosa model. This could be an explanation why the chronic bronchitis-like models secreted more CXCL-8 than normal models. Additionally, stimulation with IL-13 could also contribute by itself and not only indirectly by increasing the number of mucus-producing cells causing increased CXCL-8 production. In Paper III, the transcript expressions of inflammatory and oxidative stress genes were upregulated in PBEC-ALI/CB compared to PBEC-ALI. As mentioned above, the induced mRNA expressions of inflammatory and oxidative stress were mostly found after 6 hours of exposure to CNP in PBEC-ALI/CB, while in PBEC-ALI, the increasing effects were more pronounced after 24 hours post CNP exposure (Paper III). From these data it may be assumed that PBEC-ALI/CB generally react faster and stronger than PBEC-ALI to external stimuli. Furthermore, in Paper III, at 6 hours post CNP exposure, *MMP9* expression was significantly higher in the PBEC-ALI/CB compared to PBEC-ALI, while *TIMP1* expression was not altered. This suggest that the PBEC-ALI/CB does not counteract the increased expression of the matrix degradation enzymes after exposure to CNP, which again confirm the more susceptibility in the chronic bronchitis-like model than in the normal model. This is possibly reflecting the situation *in vivo*, where it is considered that COPD patients are more sensitivities to particulate matter in air pollutions than healthy subjects[203]. Also it has been shown that exposure to environmental stressors like ultrafine particles or nanoparticles can accelerate the progression of various chronic respiratory diseases like COPD in individuals with impaired lung function[204-206]. In line with our results, it has been shown that models built with PBEC from severely asthmatic donor produce more CXCL-8 after exposure to PM compared to models with cells from healthy donors[207]. Consistently, Kodavanti *et al*[208] reported that bronchitis rat models (SO₂ induced) exhibited increased pulmonary injury upon concentrated ambient particles exposure than the normal healthy rats. Therefore, our findings of these physiologically relevant *in vitro* models (normal and chronic bronchitis-like model), which are comparable with healthy versus pre-disposed individuals are consistent with results from *in vivo* studies that individuals with chronic bronchitis are more susceptible to exposure to particles present in air pollution.

Human bronchial epithelium together with airway macrophages serves as the first line of defense. The cooperation and communication between them play a critical role in facility reactions of the particles that enter into respiratory system.

In Paper IV, when co-cultured PBEC-ALI model with THP-1 derived macrophages (PBEC-ALI/MQ), the inflammatory response (*CXCL8*, *TNFα* and *NFKB* mRNA expression, *TIMP-1*

secretion), oxidative stress effects (*HMOX1* mRNA expression) were reduced compared with PBEC-ALI. In contrast, some studies reported in submerged culture conditions, when co-culturing primary epithelial cells or cell lines and macrophages, an amplification of the inflammatory response following exposure to ambient particulate matters or DEP was observed[209-211]. This deficiency may be due to the variance in cell types, culture conditions as well as exposure methods. When adding particles in medium as is the case when culturing under submerged condition, might lead to an agglomeration of particles. By using XposeALI exposure system which suspend the DEP into aerosolized form allowing a direct cell-particle interaction[212, 213]. The direct interaction may further induce phagocytosis of particles by MQ and initiate a protective role of MQ after DEP exposure.

Upon stimulation, macrophages can polarize into different states. Till now, two major macrophage phenotypes have been described, the classically activated type1 (M1) macrophages and the alternatively activated type 2 (M2) macrophages. Usually, M1-MQ have a pro-inflammatory role[214] while M2-MQ exert anti-inflammatory effects[33]. Hence, we speculated that the attenuated inflammatory/ oxidative stress markers expressions after DEP exposure in PBEC-ALI/MQ compared to PBEC-ALI mentioned above, could be explained by an induced polarization of macrophages to M2 phenotype with induced anti-inflammatory effects. This hypothesis was confirmed by the detection of typical M2 genes expression with anti-inflammatory properties. We found that DEP exposure upregulated the expression of M2-MQ genes (*IL4*, *MRC1*, *MCR2* and *RETNL*) in PBEC-ALI/MQ while there was no such increasing in PBEC-ALI or MQ mono-culture, and even a reduced expression of *IL13* and *RETNL* in PBEC-ALI. Besides, an induction of M2-MQ genes like *IL10*, *IL4*, *IL13* and *RETNL* was found in PBEC-ALI/MQ compared to PBEC-ALI after DEP exposure. However, in sham exposure, there was no change in M2-MQ genes expression in PBEC-ALI and PBEC-ALI/MQ were observed. Therefore, in consistence with other studies[181, 215], we assumed that cross-talk between PBEC and MQ together with DEP exposure led to M2-MQ polarization as well as anti-inflammatory effects in our multi-cellular models.

5.4 MODULATION OF TLR PATHWAY

We have previously shown a decreased TLR2 expression on blood monocytes from farmers compared to healthy controls[216]. It has also been demonstrated that smokers have a reduced expression of TLR2 in alveolar macrophages[217]. These lower TLR expressions might explain the observed increased prevalence of bacterial colonization in the respiratory tract of both smokers and farmers[216, 217]. In Paper I, we found that budesonide itself or budesonide co-stimulated with PGN/ LPS increased TLR2 mRNA expression in AMQ from smokers with

and without COPD. This finding was in line with our previous study performed on PBEC cultured submerged, that Bud combined with a TLR ligand or TNF- α synergistically enhanced PBEC TLR2 expression[52]. Glucocorticosteroids induce MKP-1 expression leading to inhibition of P38 MAPK phosphorylation and the decreased P38 activation could positively regulate TLR2 expression[218]. In addition, glucocorticosteroids increase TLR2 expression in human airway epithelial cells after incubation with *Haemophilus influenza*[218]. Furthermore, dexamethasone has been shown to increase TLR2 expression in AMQ from COPD patients[219]. Therefore, we assume that the glucocorticosteroid may enhance TLR2 expression to further strengthen immune defense pathways. Augmented TLR expression will most likely increase the possibility of PAMPs to bind to these receptors which can provide a positive feedback loop to the pro-inflammatory cytokine and chemokines secretion. This upregulation of TLR2 expression induced by budesonide appears as a paradox considering its anti-inflammatory effects (inhibit PGN/ LPS induced CXCL-8 and TNF- α release). Similar findings have been observed in dendritic cells, where glucocorticosteroids increased TLR2/TLR4 expression while decreased the production of pro-inflammatory cytokines as a response to stimulation with a TLR agonists[220]. Glucocorticosteroid exerts its anti-inflammatory functions by blocking downstream of TLR2 signaling, such as inhibiting the activation of related transcription factor (NF- κ B or AP-1). However just increasing the TLR2 expression in the upstream cannot relieve the inhibitory effects in the downstream effects caused by glucocorticosteroid[221]. Therefore, budesonide could have a dualistic effect by both increasing the host defense and reducing the inflammatory reaction. We demonstrated that in all three groups, TLR4 expression was significantly decreased when AMQ were treated with LPS alone. The same inhibitory effects of LPS on TLR4 mRNA expression were showed in AMQ from nonsmokers and smokers[222] and in a mouse macrophage cell line [223]. However, LPS was found to increase TLR4 gene expression in human neutrophils and monocytes[224].

As both bronchial epithelial cells and macrophage are equipped with TLR, also bronchial epithelial cells can modulate the inflammatory response of macrophage through TLR signaling[55]. Therefore, we investigate the effects of TLR expression when co-culturing PBEC and MQ after DEP exposure.

In Paper IV, we showed both mRNA and cell surface expression of TLR2 and TLR4 in PBEC and MQ from multicellular models after exposure to DEP. The DEP exposure increased both TLR2 and TLR4 mRNA expression in PBEC-ALI model, while there was no such alternation with the presence of MQ (PBEC-ALI/MQ). The binding of DEP to TLR2/TLR4 might activate

MyD88 or TRIF dependent pathways which increases the transcription potential of inflammatory genes[225]. Then the increased TLR expressions in PBEC-ALI upon DEP exposure, were in consisted with inflammatory biomarker findings mentioned in section 5.3 that after DEP exposure, CXCL-8 secretion/mRNA expression and *TNF α* mRNA expression were increased in PBEC-ALI but not in PBEC-ALI/MQ.

To detect cell surface TLR expression in different cell types, CD68 antibody was used to distinguish between PBEC and MQ in the PBEC-ALI/MQ model. Then it was possible to detect TLR2/ TLR4 surface expression on PBEC or MQ separately by FACS analysis. In Paper IV, we found that the surface expression of TLR2 was increased while TLR4 was decreased on both PBEC and MQ in PBEC-ALI/MQ model after DEP exposure. However, such alteration could not be found in PBEC-ALI or MQ mono-culture (except TLR4 in PBEC-ALI). Interestingly, similar pattern has been shown in Paper I, that the combination of Bud and TLR ligands increased TLR2 expression in AMQ, while TLR ligands alone decreased TLR4 expression. Besides, another study reported that alveolar macrophages, exposed to PM contaminated by low or high levels of endotoxin, activated TLR2/4 dependent pathways differently. For instance, low levels of endotoxin contamination induced TLR2 transcript expression which attenuated TLR4 pathways[226]. Conversely, in epithelial cells, PM did not change the TLR2 expression while it augmented TLR4 expression[227]. In dendritic cells, both TLR2 and TLR4 expression were downregulated by PM exposure[228]. Therefore, our results indicated that the interaction between different cell types could alter TLR expression.

Worth mentioning is that macrophage surface TLR expression is highly relevant to the macrophage polarization process. For instance, M1-MQ contains higher ratio of TLR4 and TLR2 surface expression than M2-MQ, also TLR4 deficiency can drive macrophage to skew to M2 phenotype[229, 230]. Consistently, in our study after DEP exposure, expression of macrophage surface TLR4 was attenuated in PBEC-ALI/MQ, which might strength the finding that when exposing our multi-cellular model including both PBEC and MQ to DEP an increased M2-MQ polarization was observed.

Furthermore, the FACS data regarding TLR2/4 surface expression was different from PCR data regarding TLR2/4 mRNA expression. This difference might due to that, in PBEC-ALI/MQ, the surface expression of TLR can be detected on PBEC or MQ separately by FACS, but for mRNA expression which detected by RT-PCR, PBEC and MQ cannot be analyzed separately. Moreover, FACS analysis detects TLR expression on protein level while RT-PCR detects TLR expression on mRNA level, which could also explain the differences.

6 CONCLUSIONS

Paper I:

- In alveolar macrophage (AMQ) from healthy controls and smokers with and without chronic obstructive pulmonary disease (COPD), Toll-like receptor (TLR) ligands increased secretion of pro-inflammatory cytokines and chemokines which was attenuated by a glucocorticosteroid.
- In AMQ from smokers with and without COPD, glucocorticosteroids alone or combined with TLR ligands upregulated the TLR2 mRNA expression.

Paper II:

- We successfully developed both normal and chronic bronchitis-like human bronchial epithelial 3D models by co-culturing primary bronchial epithelial cell (PBEC) with fibroblast cell line at air-liquid interface. These 3D models were combined with a unique aerosol exposures system--XposeALI.
- Both normal and chronic bronchitis-like 3D models differentiated into various cell types similar to what is found *in vivo* and chronic bronchitis-like 3D-model contained more mucus producing cells. Therefore, these models can be used to mimic *in vivo* conditions.
- 3D model displayed inflammatory responses to palladium exposure.
- The 3D model forms part of an *in vitro* testing strategy to study the cell biology, respiratory diseases, and toxicity of inhaled substances without requirement of animal studies.

Paper III:

- Carbon nanoparticle (CNP) exposure induced inflammatory and oxidative stress response in both normal (PBEC-ALI) and chronic bronchitis-like (PBEC-ALI/CB) models.
- Exposure to CNP in the normal model induced both tissue injury and repair markers while in bronchitis-like model only the tissue injury marker increased.
- There was an earlier onset with a significantly stronger reaction in the chronic bronchitis-like model compared with normal model.
- We provide further evidence for individuals with chronic bronchitis are more susceptible to exposure to particles present in air pollution.

Paper IV:

- Diesel exhaust particle (DEP) exposure increased the inflammatory and oxidative stress responses in PBEC-ALI model, and this induction was reduced by co-culturing with MQ.
- The DEP exposure increased TLR2 but decreased TLR4 surface expressions in both PBEC and MQ from PBEC-ALI/MQ.
- The co-culture of PBEC-ALI and MQ in response to DEP exposure drove the polarization of MQ to M2 phenotype, and result in efficient resolution of the inflammatory response.
- Co-cultured models enable cell-cell interactions and is therefore considered to be more effective than mono-cell culture in investigating the detailed molecular responses to particle exposure.

7 FUTURE PERSPECTIVES

In this thesis, we have established good and reliable human bronchial airway wall models which can be used as a realistic and efficient alternative for pulmonary toxicity testing and cell-cell interaction studies. Different parts of the lung may respond differently due to differences in tissue architecture structure, secretion of mucus and presence of surfactant. Therefore, in the future studies, we also want to build alveolar mucosa models.

The alveolar mucosa consists of alveolar type I and type II cells. Alveolar type I (ATI) cell which covering around 90% of the alveolar surface are usually flat and large in size. Alveolar type II (ATII) cell which display approximately 10% of the alveolar surface are usually cuboidal and smaller in size. The ATI cell plays important roles in gas exchange and ion/protein transport. The ATII cell secretes surfactant to serve as a production and also act as progenitor cells for the regeneration of ATI cell. As a response to host defense, ATII cell is involved in modulating the balance of coagulation and fibrinolysis. On one hand, ATII cell can guide neutrophils transmigrate into the alveolar space, on the other hand it can by itself act as effector cells by directly or indirectly cross-talk with resident and mobile cells.

To our knowledge there are no existing commercial ATII cell models available at present, and most of the alveolar mucosa models described in the literature are involving tumour or immobilized cell lines which might lack biological relevance, due to their genetic alterations and lacking essential physiological functions like tight junctions. Therefore, it is necessary to develop alveolar mucosa model with primary ATII cell.


All studies included in this thesis included exposure to one substance at one occasion thus reflecting an acute exposure scenario. However, repeated exposure would cause the chronic cycle of injury and repair, and significantly change the structure, gene expression and function of epithelial cells. Also in Paper IV, we observed the polarization of M2 macrophage upon diesel exhaust particle (DEP) exposure which could be speculated to be a normal defense response after acute exposure to DEP. However, the effect of chronic exposure which might induce an alteration regarding macrophage polarization as a response to a persistent inflammation is still unknown. Therefore, a more chronic exposure models would be of interest in order to detect chronic effects of different substance by repeated exposure over periods of time and also test to expose the models for a combination of particles and/or gases.


We think that our different models are suited to study disease mechanisms in detail. Therefore, we also would like to explore mechanisms of COPD and chronic bronchitis and the


mechanisms of action of established and/ or newly developed treatment strategies in our multi-cellular bronchial mucosa models.


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